Rev. 12/09/04

FEE TRANSMITTAL For FY 2005

Patent fees are subject to annual revision

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT: $ 250.00

Complete if Known	
Application Number:	09/859,701
Filing Date:	5/16/2001
First Named Inventor:	Benjamin P. Warner
Examiner Name:	Deborah A. Davis
Group/Art Unit:	1631
Attorney Docket No.:	S-94,661

METHOD OF PAYMENT (check all that apply)

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   Deposit Account Name: **Los Alamos National Laboratory**
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1203 $360	2203 $180	Multiple dependent claim, if not paid.
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FEE CALCULATION (continued)

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1809	**$790**	2809	**$395**	Filing a submission after final rejection (37 CFR 1.129 (a))	
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SUBMITTED BY

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Rev 06/04/04

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Benjamin P. Warner et al. Docket No.: S-94,661

Serial No.: 09/859,701 Examiner: Deborah A. Davis

Filed : 5/16/2001 Art Unit: 1631

For : METHOD FOR DETECTING BINDING EVENTS USING MICRO-X-RAY FLUORESCENCE SPECTROMETRY

Mail Stop Appeal Brief - Patents
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL OF APPEAL BRIEF

1. Transmitted herewith in triplicate is the Appeal Brief in this application with respect to the Notice of Appeal filed on November 30, 2004.

2. ☒ Applicant claims small entity status.

3. Attached is a Fee Transmittal Form.

Respectfully submitted,

Date: January 25, 2005

Samuel J. Borkowsky
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S-94,661

01/30/04

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Appellants:	Benjamin P. Warner et al.	Docket No.:	S-94,661
Serial No.:	09/859,701	Examiner:	Deborah A. Davis
Filed :	May 16, 2001	Art Unit:	1631

For : METHOD FOR DETECTING BINDING EVENTS USING MICRO-X-RAY FLUORESCENCE SPECTROMETRY

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Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Appellants:	Benjamin P. Warner	Docket No.:	S-94,661
Serial No.:	09/859,701	Examiner:	Deborah A. Davis
Filed :	May 16, 2001	Art Unit:	1641
For :	METHOD FOR DETECTING BINDING EVENTS USING MICRO-X-RAY FLUORESCENCE SPECTROMETRY		

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P. O. Box 1450
Alexandria, VA 22313-1450

STATEMENT OF THE REAL PARTY IN INTEREST

The Regents of the University of California is the assignee of all right, title, and interest in and to U.S. Patent Application Serial No. 09/859,701 from the Government of the United States, United States Department of Energy.

RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

STATUS OF CLAIMS

Claims 1-10 are pending in the application. Pending claims 1-10 have been finally rejected by an Office Action dated June 3, 2004. The rejection of claims 1-10 is appealed. No claims have been allowed.

STATUS OF AMENDMENTS

No amendments have been filed subsequent to the final rejection dated June 3, 2004.

SUMMARY OF INVENTION

The present invention relates to a method for detecting whether binding has occurred between chemical species. The chemical species are referred to as receptors and binders. The receptors are arrayed on a substrate before or after exposure to one

or more binders. After the receptors are either arrayed and then exposed to binders or exposed to binders and then arrayed, the present process requires that each member of the array be exposed to X-ray radiation. Any arrayed receptor having an attached binder produces a particular X-ray fluorescence signal that can be detected. The detection of that particular signal indicates that the receptor has an attached binder (i.e. a binding event has occurred).

The order of the steps is very important. Receptors must interact with binders for binding to occur. In the presently claimed invention, binding must occur before the members of the array are exposed to x-rays. The x-ray fluorescence signal that is detected is produced completely or partly from atoms in the binder. Consider, for example, applying the method to non-fluorine-containing receptors and fluorine-containing binders. Fluorine atoms produce their own characteristic x-ray fluorescence signal when they are excited by x-rays. After exposing the non-fluorine-containing receptors to the fluorine-containing binders and arraying the exposed receptors on a substrate (or arraying the receptors on a substrate and then exposing them to the binders), each member of the array would then be exposed to x-rays. The detection of an x-ray fluorescence signal from the element fluorine from any member of the array indicates that binding has occurred. Conversely, the absence of an x-ray fluorescence signal from fluorine indicates that binding between the receptor and binder has not occurred.

The following table provides a reference to specification locations that support the recited claim limitations.

Claim Limitation	Support Location
1. A method for detecting a binding event between at least one binder and members of a receptor array, comprising the steps of:	Page 4, lines 4-6 and lines 12-14; page 7, lines 23-25.
(a) exposing a plurality of receptors to at least one potential binder;	Page 4, lines 6-8 and lines 14-16; page 7, lines 25-30; page 9, lines 5-10.
(b) arraying the receptors onto a substrate;	Page 4, line 8 and lines 16-17; page 7, line 30 through page 8 line 2; page 9, lines 10-12.
(c) exposing each member of the array that has	Page 4, line 8-9 and line 17; page 8, lines 2-4; page

already been exposed to at least one potential binder to X-ray radiation to induce an X-ray fluorescence signal from each member of the array now bound to at least one binder, thereby indicating that a binding event has occurred; and	9, lines 13-20;
(d) detecting an X-ray fluorescence signal resulting from exposure to the X-ray radiation from any member of the array where a binding event has occurred.	Page 4, lines 9-10 and line 17-19; and page 8, lines 2-4; page 9, line 13 through page 10, line 8.
2. The method of claim 1, wherein the receptor comprises at least one organic compound.	Page 5, line 17 through page 6, line 2; page 6, lines 3-7; page 7, lines 27-30; page 8, lines 28-30.
3. The method of claim 1, wherein the receptor comprises at least one oligomer.	Page 5, line 17 through page 6, line 2; page 6, lines 3-7; page 7, lines 27-30; page 8, lines 28-30.
4. The method of claim 1, wherein the receptor comprises at least one polymer.	Page 5, line 17-19.
5. The method of claim 1, wherein the receptor is selected from the group consisting of esters, amines, imines, aldehydes, ketones, amides, ethers, olefins, halogenated organic molecules, antibodies, drugs, steroids, amino acids, nucleic acids, oligomers, oligonucleotides, oligosaccharides, oligopeptides, polyolefins, polyurethanes, polyesters, polycarbonates, polyamines, polyamides, halogenated polymers, polypeptides, polynucleotides, polysaccharides, nucleic acids, cell membrane receptors, viruses, cells, cellular membranes, and organelles.	Page 8, lines 4-12; page 11, lines 10-21.
6. The method of claim 1, wherein the binder comprises at least one organic molecule.	Page 7, lines 12-18.
7. The method of claim 1, wherein the binder comprises at least one oligomer.	Page 11, lines 26-27.
8. The method of claim 1, wherein the binder comprises at least one polymer.	Page 11, lines 27-30.
9. The method of claim 1, wherein the binder comprises at least one metal ion.	Page 6, lines 15-19; page 7, lines 19-22; page 11, lines 5-9; page 12, lines 2-3.
10. The method of claim 4, wherein the binder is	Page 7, lines 12-18; page 11, lines 22-30; page 12,

selected from the group consisting of esters, amines, imines, aldehydes, ketones, amides, ethers, olefins, halogenated organic molecules, antibodies, drugs, hormones, steroids, amino acids, nucleic acids, oligomers, oligonucleotides, oligosaccharides, oligopeptides, polyolefins, polyurethanes, polyesters, polycarbonates, polyamines, polyamides, halogenated polymers, polypeptides, polynucleotides, polysaccharides, nucleic acids, metal ions, anions, complex ions, oxoanions, polyoxoanions, phosphate, organophosphates, sulfate, organosulfates, zirconate, agonists and antagonists for cell membrane receptors, toxins, enzymes, enzyme substrates, cofactors, and antibodies.	lines 1-9.

ISSUES

1. Whether claims 1-8 and 10 were properly rejected under 35 U.S.C. 102(b) as being anticipated by Pirrung et al (WO 90/15070).

2. Whether claim 9 was properly rejected under 35 U.S.C. 103(a) over Pirrung et al. in view of Weinberg et al (U.S. Patent 6,030,917).

GROUPING OF CLAIMS

Applicants submit that claims 2-8 and 10 are dependent upon claim 1 and their patentability under 35 U.S.C. 102(b) will stand or fall with independent claim 1. Claim 9 is dependent on claim 1 and its patentability will also stand or fall with independent claim 1.

ARGUMENTS

1. Claims 1-8 and 10 are not anticipated by Pirrung et al.

According to the Office Action of June 3, 2004, the final rejection under 35 U.S. C. §102(b) of claims 1-8 and 10 was based on Pirrung et al. The Office Action states that "...Pirrung et al. teaches a method and device for preparing desired sequences on a substrate at known locations wherein bound material of the substrate is exposed to irradiation (pg. 10, lines 1-35) so as to activate material and permit binding (see

abstract). The substrate has a variety of uses such as screening large numbers of peptides or receptors, wherein receptors are labeled with fluorescent markers for detection...". According to the Office Action, "...the substrate of Pirrung et al. is irradiated to remove protecting groups that interfere with binding activity. After removing the protecting groups, the substrates are exposed to one or more receptors and screened for biological (binding) activity. The biological activity is analyzed by photon detection (x-ray radiation), autoradiographic or visible light techniques to induce a signal (page 5, lines 14-30)...". According to the Office Action, "...not only does Pirrung et al teach potential binders that are tagged and detected through visible light, but potential binders are also detected through photon excitation (X-ray) and autoradiographic techniques...".

Appellant's invention is a method for measuring the binding between a plurality of receptors and one or more binders using x-ray fluorescence. Appellant's method is claimed explicitly in claim 1 as follows:

(a) exposing a plurality of receptors to at least one potential binder;

(b) arraying the receptors onto a substrate;

(c) exposing each member of the array that has already been exposed to at least one potential binder to X-ray radiation to induce an X-ray fluorescence signal from each member of the array now bound to at least one binder, thereby indicating that a binding event has occurred; and

(d) detecting an X-ray fluorescence signal resulting from exposure to the X-ray radiation from any member of the array where a binding event has occurred.

Pirrung et al. use terminology that is different from Appellant. A comparison of claim 1 of Pirrung et al. with claim 1 of Appellant's present patent application indicates that Pirrung et al. gives the term "substrate" a meaning similar to Appellant's term "receptor". A comparison of claim 6 of Pirrung et al. with Appellant's claim 1 indicates that Pirrung et al. gives the term "receptor" a meaning similar to Appellant's term "binder".

Pirrung uses x-ray irradiation to remove protecting groups that interfere with binding. If any binding occurs between an arrayed receptor and a binder, Pirrung et al. requires that it occur after x-ray irradiation. By contrast, Appellant's claimed invention includes "...exposing each member of the array that has already been exposed to at least one potential binder to X-ray radiation to induce an X-ray fluorescence signal from each member of the array now bound to at least one binder...". Thus, binders are exposed to receptors before x-ray irradiation exposure. In fact, Appellant's invention becomes inoperable if receptors were exposed to binders after x-ray irradiation because the detected x-ray fluorescence signal is completely or at least partly due to x-ray emission from any binder attached to a receptor. The binder must be attached to a receptor before x-radiation and x-ray fluorescence detection. Thus, the order of the steps is critical for both Appellant and Pirrung et al., and the Appellant's required order as claimed in claim 1 above is different from the order required by Pirrung et al. For this reason alone, claim 1 of Appellant's present patent application is not anticipated by Pirrung et al.

Additionally, Appellant's claimed invention is not anticipated by Pirrung et al. because Appellant uses x-ray fluorescence detection to detect binding and Pirrung et al. does not. X-ray fluorescence occurs when an atom is ionized using externally applied x-ray radiation. X-ray exposure results in the ejection of an inner core electron from the atom. The result is an ion in an excited state. The ion relaxes to the ground state by the electronic emission of an x-ray photon. This type of emission is different from other forms of X-ray emission, such as the x-ray emission that occurs during the nuclear decay of a radioactive atom. X-ray fluorescence takes place only after x-ray exposure, while x-ray emission during nuclear emission occurs spontaneously for radioactive elements. Importantly, the energies of x-ray fluorescence photons are different from the energies of x-rays photons emitted by radioactive atoms during nuclear decay. While Pirrung et al. does use x-rays, they are used to remove protecting groups and not to induce x-ray fluorescence signals. Importantly, Pirrung et al. does not detect any x-ray fluorescence signals. Any x-ray fluorescence signal that Pirrung et al. would detect could not be used to indicate binding because no binders are present at

the time. Pirrung et al. does mention fluorescence detection and describes using fluorescent markers, which are chemical groups attached to the binder that emit visible or near-ultraviolet light when exposed to visible or ultraviolet light. Pirrung et al. is using these fluorescent markers to detect optical visible or ultraviolet fluorescence, not x-ray fluorescence. Pirrung et al. also mentions using autoradiography, which involves the emission of x-rays and/or gamma rays during nuclear decay. As Appellant has explained, X-ray fluorescence requires that a sample be exposed to external x-ray irradiation to induce ionization and subsequent electronic x-ray emission from the sample. By contrast, autoradiography does not involve exposing a sample to x-rays to induce ionization and subsequent x-ray emission. In autoradiography, x-ray emission occurs naturally because the sample includes radioactive elements that spontaneously emit x-rays and/or gamma rays from the nucleus. Thus, autoradiography is significantly different from x-ray fluorescence.

In the present patent application, Appellant explained that a significant problem associated with fluorescent tags (i.e. fluorescent markers) is that any binder modified with an attached marker does not necessarily have the same binding properties as the unmodified, original binder. This is the most significant problem that Appellant's invention addresses. Appellant's invention does not require the attachment of any type of marker, and the attachment of a marker to a binder is discouraged because the marker could change the binding properties of the binder. A modified binder might not bind to a receptor, but the unmodified analog might. Similarly, a modified binder might bind to the receptor while the unmodified analog might not. The strength of the binding could be also affected by the addition of the marker. So it is possible that the use of these markers can change the binding properties of the binder. Pirrung et al. requires some type of marker. Appellant does not.

Additionally, Appellant's claimed invention is not obvious in view of Pirrung et al. Pirrung et al. provides no motivation or suggestion to change the order of the steps of the Pirrung et al. method to arrive at Appellant's claimed invention as claimed in claim 1 of Appellant's present patent application.

In summary, Pirrung et al. does not teach Appellant's claimed invention. Claims 1-8 and 10 are neither anticipated by, nor obvious in view of, Pirrung et al. In view of the clear teachings of the application, the rejection of Claims 1-8 and 10 under 35 U.S.C. §102(b) are urged to be reversed.

2. Claim 9 is not obvious over the references Pirrung et al. and Weinberg et al.

According to the Office Action of June 3, 2004, the final rejection under 35 U.S. C. §103(a) of claim 9 was based on Pirrung et al. in view of Weinberg et al. According to the Office Action, while Pirrung et al. is silent with respect to metal ion binders, Weinberg et al. teaches methods of screening and characterization of libraries of organometallic compounds that can be used as catalysts and therapeutic agents. The Office Action also states that ancillary ligand-stabilized metal complexes are also useful as catalysts for reactions such as oxidation, reduction, hydrogenation, polymerization, carbonylation, and other reactions, and that it would have been obvious to one of ordinary skill in the art to use the metal ion binder of Weinberg et al. in the method of Pirrung et al. and device for preparing desired sequences on a substrate as taught by Pirrung et al. to screen for therapeutic agents and catalysts that are useful in oxidation, reduction, and other useful reactions.

Appellant submits that there are important differences between Appellant's claimed method and the claimed method of Pirrung et al. Neither Pirrung et al. nor Weinberg et al. provide any motivation or suggestion for their combination. Even if there were such a motivation or suggestion in either of these references, Weinberg et al. does not describe modifying the teachings of Pirrung et al. for exposing the substrate array of Pirrung et al. to binders before the array is exposed to x-ray radiation. Weinberg et al. also does not describe modifying the teachings of Pirrung et al. with regard to detecting binding using an x-ray fluorescence signal. In view of the clear teachings of Appellant's application, the rejection of claim 9 under 35 U.S.C. §103(a) is urged to be reversed.

CONCLUSION

In view of the arguments presented herein above, Appellants submit that Pirrung et al., which has been applied in the rejection of claims 1-8 and 10 under 35 U.S.C.

102(b), does not anticipate, or render obvious, the subject claimed invention. Appellants also submit that the combination of Pirrung et al. and Weinberg et al, which have been applied in the rejection of claim 9 under 35 U.S.C. 103(a), do not render obvious the subject claimed invention. Applicants have clearly described and claimed a method in a manner that is different and non-obvious from that of the references cited alone and in combination. The rejections of claims 1-10 should therefore be reversed and the claims on appeal remanded to the Examiner for further appropriate action.

Respectfully submitted,

Date: January 24 2005

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Signature of Agent

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APPENDIX A - CLAIMS ON APPEAL

1. A method for detecting a binding event between at least one binder and members of a receptor array, comprising the steps of:

(d) exposing a plurality of receptors to at least one potential binder;

(e) arraying the receptors onto a substrate;

(f) exposing each member of the array that has already been exposed to at least one potential binder to X-ray radiation to induce an X-ray fluorescence signal from each member of the array now bound to at least one binder, thereby indicating that a binding event has occurred; and

(g) detecting an X-ray fluorescence signal resulting from exposure to the X-ray radiation from any member of the array where a binding event has occurred.

2. The method of claim 1, wherein the receptor comprises at least one organic compound.

3. The method of claim 1, wherein the receptor comprises at least one oligomer.

4. The method of claim 1, wherein the receptor comprises at least one polymer.

5. The method of claim 1, wherein the receptor is selected from the group consisting of esters, amines, imines, aldehydes, ketones, amides, ethers, olefins, halogenated organic molecules, antibodies, drugs, steroids, amino acids, nucleic acids, oligomers, oligonucleotides, oligosaccharides, oligopeptides, polyolefins, polyurethanes, polyesters, polycarbonates, polyamines, polyamides, halogenated polymers, polypeptides, polynucleotides, polysaccharides, nucleic acids, cell membrane receptors, viruses, cells, cellular membranes, and organelles.

6. The method of claim 1, wherein the binder comprises at least one organic molecule.

7. The method of claim 1, wherein the binder comprises at least one oligomer.

8. The method of claim 1, wherein the binder comprises at least one polymer.

9. The method of claim 1, wherein the binder comprises at least one metal ion.

10. The method of claim 4, wherein the binder is selected from the group consisting of esters, amines, imines, aldehydes, ketones, amides, ethers, olefins, halogenated organic molecules, antibodies, drugs, hormones, steroids, amino acids, nucleic acids, oligomers, oligonucleotides, oligosaccharides, oligopeptides, polyolefins, polyurethanes, polyesters, polycarbonates, polyamines, polyamides, halogenated polymers, polypeptides, polynucleotides, polysaccharides, nucleic acids, metal ions, anions, complex ions, oxoanions, polyoxoanions, phosphate, organophosphates, sulfate, organosulfates, zirconate, agonists and antagonists for cell membrane receptors, toxins, enzymes, enzyme substrates, cofactors, and antibodies.

APPENDIX B
CLAIM COMPARISON WITH REJECTION

Claim limitation	Examiner's comment	Reference citation	Appellant's comment
1. A method for detecting a binding event between at least one binder and members of a receptor array, comprising the steps of:	Pirrung et al. teaches a method and device for preparing desired sequences on a substrate at known locations wherein bound material of the substrate is exposed to irradiation (pg. 10, lines 1-35) so as to activate material and permit binding (see abstract). The substrate has a wide variety of uses such as screening large numbers of peptides or receptors, wherein receptors are labeled with fluorescent markers for detection. Other applications of the invention include doping of organic material in the substrate (pg. 5, lines 14-36). In an alternative embodiment the surface may comprise cage binding members that are capable of immobilizing receptors in predefined regions of a substrate for selective activation that allow receptors that have differential affinity for one or more ligands to react (pg. 55, lines 30-37) and pg. 56. lines 1-11). A specific binding substance having strong binding affinity for the binding member and a strong affinity for the receptor or a conjugate of the receptor may be used to act as a bridge between binding members and receptors if desired. The method uses a receptor prepared such that the	According the Pirrung et al. Abstract, Pirrung et al. teaches a method and device for preparing sequences on a substrate at known locations. Known locations of a substrate are irradiated by way of a mask so as to activate a material for binding. The substrate is then exposed to a first material for binding thereto. Second locations are then irradiated through a mask and exposed to a second material. A variety of sequences may be formed through selective irradiation of the substrate followed by application of selected materials.	Appellant's invention is a method for measuring the binding between a plurality of receptors and one or more binders using x-ray fluorescence. Pirrung et al. teaches the use of x-rays to activate a plurality of receptors to bind to a binder. Appellant requires that any binding take place before irradiation. Appellant's invention is inoperable if the irradiation occurs before receptors are exposed to binders. Pirrung et al. requires that any binding take place after X-ray irradiation, and Pirrung et al. does not teach irradiation after binding.

	receptor retains its activity toward a particular ligand (pg. 56, lines 30-36). According to Pirrung et al, receptors used in this method could be organic compounds such as polymers (oligomer), nucleic acids, peptides, drugs, cellular membranes cells, etc. (pg. 11, lines 7-24). The binder molecule can be selected from the group consisting of agonists and antagonists for cell membrane receptors, oligonucleotides, nucleic acids, proteins, antibodies, etc. (pg. 9, lines 30-37).		
(a) exposing a plurality of receptors to at least one potential binder;	Large numbers of peptides or receptors are screened. The receptors are labeled with fluorescent markers for detection.	Page 5, lines 5-36 discloses screening large numbers of peptides or receptors, wherein the receptors are labeled with fluorescent markers for detection.	Pirrung et al. receptors are not the equivalent of Appellant's receptors; they are the equivalent of Appellant's binders. Appellant's steps have a specific order. Receptors are exposed to one or more potential binders (step a) and are arrayed onto a substrate (step b) before exposure to x-ray radiation (step c). Pirrung et al. exposes the substrate array to x-rays first in order to remove protective groups, and then exposes the de-protected array to potential binders.
(b) arraying the receptors onto a substrate;	Pirrung et al. teaches a method and device for preparing desired sequences on a substrate at known locations.	According to the abstract, Pirrung et al. teaches the preparation of sequences on a substrate at known locations.	Both Pirrung et al. and Appellant use an array of receptors.
(c) exposing each member of the array that has already been exposed to at least	The substrate of Pirrung et al. is irradiated to remove protecting groups that interfere with binding	Pirrung et al., page 59, claim 2 teaches exposing a region of a substrate to an activator to remove	Appellant's steps have a specific order. Receptors are exposed to one or more potential binders

one potential binder to X-ray radiation to induce an X-ray fluorescence signal from each member of the array now bound to at least one binder, thereby indicating that a binding event has occurred; and	activity. After removing protecting groups, the substrates are exposed to one or more receptors and screened for biological activity.	a protective group, where the activator can be x-rays. The Abstract of Pirrung et al. teaches the order of the steps, and that the x-ray exposure takes place before exposure to a material for binding. There is no teaching in Pirrung et al. to expose each member of the array to x-rays after each member has been exposed to a binder.	(step a) and are arrayed onto a substrate (step b) before exposure to x-ray radiation (step c). Pirrung et al. exposes the substrate array to x-rays first in order to remove protective groups, and then exposes the de-protected array to potential binders.
(d) detecting an X-ray fluorescence signal resulting from exposure to the X-ray radiation from any member of the array where a binding event has occurred.	The biological activity is analyzed by photon detection (x-ray radiation), autoradiographic or visible light techniques to induce a signal (page 5, lines 14-30). Therefore, not only does Pirrung et al. teach potential binders that are tagged and detected through visible light, but potential binders are also detected through photon excitation (x-ray) and autoradiographic techniques).	There is no teaching in Pirrung et al. related to detecting an x-ray fluorescence signal.	Appellant teaches x-ray fluorescence detection. Pirrung et al. does not teach x-ray fluorescence detection, or more generally, x-ray fluorescence.

APPENDIX C
PCT Application WO 90/15070

APPENDIX D
U.S. Patent 6,030,917

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 1/04, 17/06, 17/14
B01J 19/00

A1

(11) International Publication Number: WO 90/15070

(43) International Publication Date: 13 December 1990 (13.12.90)

(21) International Application Number: PCT/NL90/00081

(22) International Filing Date: 7 June 1990 (07.06.90)

(30) Priority data:

362,901	7 June 1989 (07.06.89)	US
492,462	7 March 1990 (07.03.90)	US

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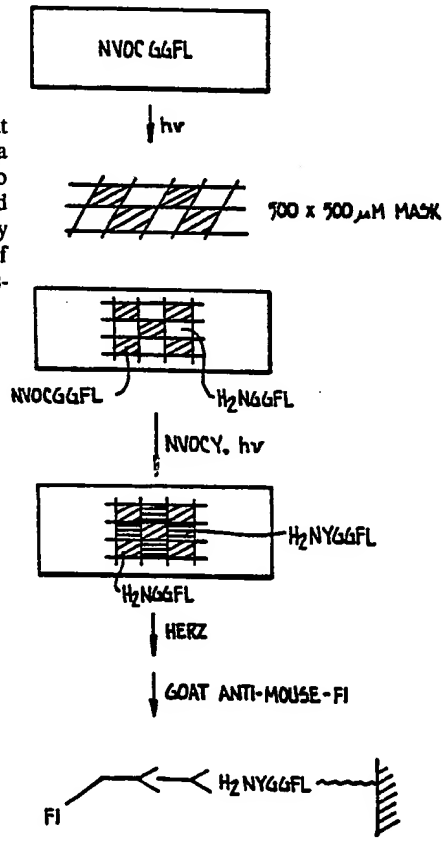
(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE*, DE (European patent)*, DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: VERY LARGE SCALE IMMOBILIZED PEPTIDE SYNTHESIS

(57) Abstract

A method and device for preparing desired sequences on a substrate at known locations. Known locations (10) of a substrate (2) are irradiated by way of a mask (8) so as to activate a material (4) for binding. The substrate is then exposed to a first material (12) for binding thereto. Second locations (14) are then irradiated through a mask and exposed to a second material (16). A variety of sequences may be formed through selective irradiation of the substrate followed by application of selected materials. A reactor system and fluorescence detection system are also disclosed.



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VERY LARGE SCALE IMMOBILIZED PEPTIDE SYNTHESIS

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BACKGROUND OF THE INVENTION

The present inventions relate to the synthesis and placement materials at known locations. In particular, one embodiment of the inventions provides a method and associated apparatus for preparing diverse chemical sequences at known locations on a single substrate surface. The inventions may be applied, for example, in the field of preparation of oligomer, peptide, nucleic acid, oligosaccharide, phospholipid, polymer, or drug congener preparation, especially to create sources of chemical diversity for use in screening for biological activity.

The relationship between structure and activity of molecules is a fundamental issue in the study of biological systems. Structure-activity relationships are important in understanding, for example, the function of enzymes, the ways in which cells communicate with each other, as well as cellular control and feedback systems.

Certain macromolecules are known to interact and bind to other molecules having a very specific three-dimensional spatial and electronic distribution. Any large molecule having such specificity can be considered a receptor, whether it is an enzyme catalyzing hydrolysis of a metabolic intermediate, a cell-surface protein mediating membrane transport of ions, a glycoprotein serving to identify a particular cell to its neighbors,

an IgG-class antibody circulating in the plasma, an oligonucleotide sequence of DNA in the nucleus, or the like. The various molecules which receptors selectively bind are known as ligands.

Many assays are available for measuring the binding affinity of known receptors and ligands, but the information which can be gained from such experiments is often limited by the number and type of ligands which are available. Novel ligands are sometimes discovered by chance or by application of new techniques for the elucidation of molecular structure, including x-ray crystallographic analysis and recombinant genetic techniques for proteins.

Small peptides are an exemplary system for exploring the relationship between structure and function in biology. A peptide is a sequence of amino acids. When the twenty naturally occurring amino acids are condensed into polymeric molecules they form a wide variety of three-dimensional configurations, each resulting from a particular amino acid sequence and solvent condition. The number of possible pentapeptides of the 20 naturally occurring amino acids, for example, is 20^5 or 3.2 million different peptides. The likelihood that molecules of this size might be useful in receptor-binding studies is supported by epitope analysis studies showing that some antibodies recognize sequences as short as a few amino acids with high specificity. Furthermore, the average molecular weight of amino acids puts small peptides in the size range of many currently useful pharmaceutical products.

Pharmaceutical drug discovery is one type of research which relies on such a study of structure-activity relationships. In most cases, contemporary pharmaceutical research can be described as the process of discovering novel ligands with desirable patterns of specificity for biologically important receptors.

Another example is research to discover new compounds for use in agriculture, such as pesticides and herbicides.

Sometimes, the solution to a rational process of designing ligands is difficult or unyielding. Prior methods of preparing large numbers of different polymers have been painstakingly slow when used at a scale sufficient to permit effective rational or random screening. For example, the "Merrifield" method (J. Am. Chem. Soc. (1963) 85:2149-2154, which is incorporated herein by reference for all purposes) has been used to synthesize peptides on a solid support. In the Merrifield method, an amino acid is covalently bonded to a support made of an insoluble polymer. Another amino acid with an alpha protected group is reacted with the covalently bonded amino acid to form a dipeptide. After washing, the protective group is removed and a third amino acid with an alpha protective group is added to the dipeptide. This process is continued until a peptide of a desired length and sequence is obtained. Using the Merrifield method, it is not economically practical to synthesize more than a handful of peptide sequences in a day.

To synthesize larger numbers of polymer sequences, it has also been proposed to use a series of reaction vessels for polymer synthesis. For example, a tubular reactor system may be used to synthesize a linear polymer on a solid phase support by automated sequential addition of reagents. This method still does not enable the synthesis of a sufficiently large number of polymer sequences for effective economical screening.

Methods of preparing a plurality of polymer sequences are also known in which a foraminous container encloses a known quantity of reactive particles, the particles being larger in size than foramina of the container. The containers may be selectively reacted with desired materials to synthesize desired sequences of product molecules. As with other methods known in the

art, this method cannot practically be used to synthesize a sufficient variety of polypeptides for effective screening.

Other techniques have also been described. These methods include the synthesis of peptides on 96 plastic pins which fit the format of standard microtiter plates. Unfortunately, while these techniques have been somewhat useful, substantial problems remain. For example, these methods continue to be limited in the diversity of sequences which can be economically synthesized and screened.

From the above, it is seen that an improved method and apparatus for synthesizing a variety of chemical sequences at known locations is desired.

SUMMARY OF THE INVENTION

An improved method and apparatus for the preparation of a variety of polymers is disclosed.

In one preferred embodiment, linker molecules are provided on a substrate. A terminal end of the linker molecules is provided with a reactive functional group protected with a photoremovable protective group. Using lithographic methods, the photoremovable protective group is exposed to light and removed from the linker molecules in first selected regions. The substrate is then washed or otherwise contacted with a first monomer that reacts with exposed functional groups on the linker molecules. In a preferred embodiment, the monomer is an amino acid containing a photoremovable protective group at its amino or carboxy terminus and the linker molecule terminates in an amino or carboxy acid group bearing a photoremovable protective group.

A second set of selected regions is, thereafter, exposed to light and the photoremovable protective group on the linker molecule/protected amino acid is removed at the second set of regions. The substrate is then contacted with a second monomer

containing a photoremovable protective group for reaction with exposed functional groups. This process is repeated to selectively apply monomers until polymers of a desired length and desired chemical sequence are obtained. Photolabile groups are then optionally removed and the sequence is, thereafter, optionally capped. Side chain protective groups, if present, are also removed.

By using the lithographic techniques disclosed herein, it is possible to direct light to relatively small and precisely known locations on the substrate. It is, therefore, possible to synthesize polymers of a known chemical sequence at known locations on the substrate.

The resulting substrate will have a variety of uses including, for example, screening large numbers of polymers for biological activity. To screen for biological activity, the substrate is exposed to one or more receptors such as antibody whole cells, receptors on vesicles, lipids, or any one of a variety of other receptors. The receptors are preferably labeled with, for example, a fluorescent marker, radioactive marker, or a labeled antibody reactive with the receptor. The location of the marker on the substrate is detected with, for example, photon detection or autoradiographic techniques. Through knowledge of the sequence of the material at the location where binding is detected, it is possible to quickly determine which sequence binds with the receptor and, therefore, the technique can be used to screen large numbers of peptides. Other possible applications of the inventions herein include diagnostics in which various antibodies for particular receptors would be placed on a substrate and, for example, blood sera would be screened for immune deficiencies. Still further applications include, for example, selective "doping" of organic materials in semiconductor devices, and the like.

In connection with one aspect of the invention an improved reactor system for synthesizing polymers is also disclosed. The reactor system includes a substrate mount which engages a substrate around a periphery thereof. The substrate mount provides for a reactor space between the substrate and the mount through or into which reaction fluids are pumped or flowed. A mask is placed on or focused on the substrate and illuminated so as to deprotect selected regions of the substrate in the reactor space. A monomer is pumped through the reactor space or otherwise contacted with the substrate and reacts with the deprotected regions. By selectively deprotecting regions on the substrate and flowing predetermined monomers through the reactor space, desired polymers at known locations may be synthesized.

Improved detection apparatus and methods are also disclosed. The detection method and apparatus utilize a substrate having a large variety of polymer sequences at known locations on a surface thereof. The substrate is exposed to a fluorescently labeled receptor which binds to one or more of the polymer sequences. The substrate is placed in a microscope detection apparatus for identification of locations where binding takes place. The microscope detection apparatus includes a monochromatic or polychromatic light source for directing light at the substrate, means for detecting fluoresced light from the substrate, and means for determining a location of the fluoresced light. The means for detecting light fluoresced on the substrate may in some embodiments include a photon counter. The means for determining a location of the fluoresced light may include an x/y translation table for the substrate. Translation of the slide and data collection are recorded and managed by an appropriately programmed digital computer.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 illustrates masking and irradiation of a substrate at a first location. The substrate is shown in cross-section;

Fig. 2 illustrates the substrate after application of a monomer "A";

Fig. 3 illustrates irradiation of the substrate at a second location;

Fig. 4 illustrates the substrate after application of monomer "B";

Fig. 5 illustrates irradiation of the "A" monomer;

Fig. 6 illustrates the substrate after a second application of "B";

Fig. 7 illustrates a completed substrate;

Figs. 8A and 8B illustrate alternative embodiments of a reactor system for forming a plurality of polymers on a substrate;

Fig. 9 illustrates a detection apparatus for locating fluorescent markers on the substrate;

Figs. 10A-10M illustrate the method as it is applied to the production of the trimers of monomers "A" and "B";

Figs. 11A, 11B, and 11C are fluorescence traces for standard fluorescent beads;

Figs. 12A and 12B are fluorescence curves for NVOC slides not exposed and exposed to light respectively;

Figs. 13A and 13B illustrate formation of a slide with a checkerboard pattern of YGGFL and GGFL exposed to labeled Herz antibody; and

Figs. 14A and 14B illustrate the mapping of sixteen sequences synthesized on two different glass slides.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

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I. Glossary

The following terms are intended to have the following general meanings as they are used herein:

1. Complementary: Refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor. Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

2. Epitope: The portion of an antigen molecule which is delineated by the area of interaction with the subclass of receptors known as antibodies.

3. Ligand: A ligand is a molecule that is recognized by a particular receptor. Examples of ligands that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars,

oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

4. Monomer: A member of the set of small molecules which can be joined together to form a polymer. The set of monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic amino acids, the set of nucleotides and the set of pentoses and hexoses. As used herein, monomers refers to any member of a basis set for synthesis of a polymer. For example, dimers of L-amino acids form a basis set of 400 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer.

5. Peptide: A polymer in which the monomers are alpha amino acids and which are joined together through amide bonds and alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer. Peptides are more than two amino acid monomers long, and often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, Biochemstry, Third Ed., 1988, which is incorporated herein by reference for all purposes.

6. Radiation: Energy which may be selectively applied including energy having a wavelength of between 10^{-14} and 10^{4} meters including, for example, electron beam radiation, gamma radiation, x-ray radiation, ultra-violet radiation, visible light, infrared radiation,

microwave radiation, and radio waves. "Irradiation" refers to the application of radiation to a surface.

7. Receptor: A molecule that has an affinity for a given ligand. Receptors may be naturally-occuring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the term receptors is used herein, no difference in meaning is intended. A "Ligand Receptor Pair" is formed when two macromolecules have combined through molecular recognition to form a complex.

Other examples of receptors which can be investigated by this invention include but are not restricted to:

a) Microorganism receptors: Determination of ligands which bind to receptors, such as specific transport proteins or enzymes essential to survival of microorganisms, is useful in a new class of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and those bacteria resistant to the antibiotics in current use.

b) Enzymes: For instance, the binding site of enzymes such as the enzymes responsible for cleaving neurotransmitters; determination of ligands which bind to certain receptors to modulate the action of the enzymes which cleave the different neurotransmitters is useful in the development of drugs which can be used in the treatment of disorders of neurotransmission.

c) Antibodies: For instance, the invention may be useful in investigating the ligand-binding site on the antibody molecule which combines with the epitope of an antigen of interest; determining a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases (e.g., by blocking the binding of the "self" antibodies).

d) Nucleic Acids: Sequences of nucleic acids may be synthesized to establish DNA or RNA binding sequences.

e) Catalytic Polypeptides: Polymers, preferably polypeptides, which are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, which functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides are described in, for example, U.S. application Serial No. 404,920, which

is incorporated herein by reference for all purposes.

f) Hormone receptors: For instance, the receptors for insulin and growth hormone. Determination of the ligands which bind with high affinity to a receptor is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes, and in the other case, a replacement for the scarce human growth hormone which can only be obtained from cadavers or by recombinant DNA technology. Other examples are the vasoconstrictive hormone receptors; determination of those ligands which bind to a receptor may lead to the development of drugs to control blood pressure.

g) Opiate receptors: Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

8. Substrate: A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. According to other embodiments, small beads may be provided on the surface which may be released upon completion of the synthesis.

9. Protective Group: A material which is bound to a monomer unit and which may be spatially removed upon selective exposure to an activator such as electromagnetic radiation. Examples of protective groups with utility herein include Nitroveratryloxy

carbonyl, Nitrobenzyloxy carbonyl, Dimethyl dimethoxybenzyloxy carbonyl, 5-Bromo-7-nitroindolinyl, *o*-Hydroxy-α-methyl cinnamoyl, and 2-Oxymethylene anthraquinone. Other examples of activators include ion beams, electric fields, magnetic fields, electron beams, x-ray, and the like.

10. Predefined Region: A predefined region is a localized area on a surface which is, was, or is intended to be activated for formation of a polymer. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions."

11. Substantially Pure: A polymer is considered to be "substantially pure" within a predefined region of a substrate when it exhibits characteristics that distinguish it from other predefined regions. Typically, purity will be measured in terms of biological activity or function as a result of uniform sequence. Such characteristics will typically be measured by way of binding with a selected ligand or receptor.

II. General

The present invention provides methods and apparatus for the preparation and use of a substrate having a plurality of polymer sequences in predefined regions. The invention is described herein primarily with regard to the preparation of molecules containing sequences of amino acids, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and

peptides having either α-, β-, or ω-amino acids, hetero-polymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. In a preferred embodiment, the invention herein is used in the synthesis of peptides.

The prepared substrate may, for example, be used in screening a variety of polymers as ligands for binding with a receptor, although it will be apparent that the invention could be used for the synthesis of a receptor for binding with a ligand. The substrate disclosed herein will have a wide variety of other uses. Merely by way of example, the invention herein can be used in determining peptide and nucleic acid sequences which bind to proteins, finding sequence-specific binding drugs, identifying epitopes recognized by antibodies, and evaluation of a variety of drugs for clinical and diagnostic applications, as well as combinations of the above.

The invention preferably provides for the use of a substrate "S" with a surface. Linker molecules "L" are optionally provided on a surface of the substrate. The purpose of the linker molecules, in some embodiments, is to facilitate receptor recognition of the synthesized polymers.

Optionally, the linker molecules may be chemically protected for storage purposes. A chemical storage protective group such as t-BOC (t-butoxycarbonyl) may be used in some embodiments. Such chemical protective groups would be chemically removed upon exposure to, for example, acidic solution and would serve to protect the surface during storage and be removed prior to polymer preparation.

On the substrate or a distal end of the linker molecules, a functional group with a protective group P_0

is provided. The protective group P_0 may be removed upon exposure to radiation, electric fields, electric currents, or other activators to expose the functional group.

In a preferred embodiment, the radiation is ultraviolet (UV), infrared (IR), or visible light. As more fully described below, the protective group may alternatively be an electrochemically-sensitive group which may be removed in the presence of an electric field. In still further alternative embodiments, ion beams, electron beams, or the like may be used for deprotection.

In some embodiments, the exposed regions and, therefore, the area upon which each distinct polymer sequence is synthesized are smaller than about 1 cm^2 or less than 1 mm^2. In preferred embodiments the exposed area is less than about 10,000 μm^2 or, more preferably, less than 100 μm^2 and may, in some embodiments, encompass the binding site for as few as a single molecule. Within these regions, each polymer is preferably synthesized in a substantially pure form.

Concurrently or after exposure of a known region of the substrate to light, the surface is contacted with a first monomer unit M_1 which reacts with the functional group which has been exposed by the deprotection step. The first monomer includes a protective group P_1. P_1 may or may not be the same as P_0.

Accordingly, after a first cycle, known first regions of the surface may comprise the sequence:

$$S\text{-}L\text{-}M_1\text{-}P_1$$

while remaining regions of the surface comprise the sequence:

$$S\text{-}L\text{-}P_0.$$

Thereafter, second regions of the surface (which may include the first region) are exposed to light and contacted with a second monomer M_2 (which may or may not be the same as M_1) having a protective group P_2. P_2 may or may not be the same as P_0 and P_1. After this second cycle, different regions of the substrate may comprise one or more of the following sequences:

$$S\text{-}L\text{-}M_1\text{-}M_2\text{-}P_2$$
$$S\text{-}L\text{-}M_2\text{-}P_2$$
$$S\text{-}L\text{-}M_1\text{-}P_1 \text{ and/or}$$
$$S\text{-}L\text{-}P_0.$$

The above process is repeated until the substrate includes desired polymers of desired lengths. By controlling the locations of the substrate exposed to light and the reagents exposed to the substrate following exposure, the location of each sequence will be known.

Thereafter, the protective groups are removed from some or all of the substrate and the sequences are, optionally, capped with a capping unit C. The process results in a substrate having a surface with a plurality of polymers of the following general formula:

$$S\text{-}[L]\text{-}(M_i)\text{-}(M_j)\text{-}(M_k)\ldots(M_x)\text{-}[C]$$

where square brackets indicate optional groups, and $M_i \ldots M_x$ indicates any sequence of monomers. The number of monomers could cover a wide variety of values, but in a preferred embodiment they will range from 2 to 100.

In some embodiments a plurality of locations on the substrate polymers are to contain a common monomer subsequence. For example, it may be desired to synthesize a sequence $S\text{-}M_1\text{-}M_2\text{-}M_3$ at first locations and a sequence $S\text{-}M_4\text{-}M_2\text{-}M_3$ at second locations. The process would commence with irradiation of the first locations

followed by contacting with M_1-P, resulting in the sequence S-M_1-P at the first location. The second locations would then be irradiated and contacted with M_4-P, resulting in the sequence S-M_4-P at the second locations. Thereafter both the first and second locations would be irradiated and contacted with the dimer M_2-M_3, resulting in the sequence S-M_1-M_2-M_3 at the first locations and S-M_4-M_2-M_3 at the second locations. Of course, common subsequences of any length could be utilized including those in a range of 2 or more monomers, 2 to 100 monomers, 2 to 20 monomers, and a most preferred range of 2 to 3 monomers.

According to other embodiments, a set of masks is used for the first monomer layer and, thereafter, varied light wavelengths are used for selective deprotection. For example, in the process discussed above, first regions are first exposed through a mask and reacted with a first monomer having a first protective group P_1, which is removable upon exposure to a first wavelength of light (e.g., IR). Second regions are masked and reacted with a second monomer having a second protecive group P_2, which is removable upon exposure to a second wavelength of light (e.g., UV). Thereafter, masks become unnecessary in the synthesis because the entire substrate may be exposed alternatively to the first and second wavelengths of light in the deprotection cycle.

The polymers prepared on a substrate according to the above methods will have a variety of uses including, for example, screening for biological activity. In such screening activities, the substrate containing the sequences is exposed to an unlabeled or labeled receptor such as an antibody, receptor on a cell, phospholipid vesicle, or any one of a variety of other receptors. In one preferred embodiment the polymers are exposed to a first, unlabeled receptor of interest and, thereafter, exposed to a labeled receptor-specific recognition element, which is, for example, an antibody. This

process will provide signal amplification in the detection stage.

The receptor molecules may bind with one or more polymers on the substrate. The presence of the labeled receptor and, therefore, the presence of a sequence which binds with the receptor is detected in a preferred embodiment through the use of autoradiography, detection of fluorescence with a charge-coupled device, fluorescence microscopy, or the like. The sequence of the polymer at the locations where the receptor binding is detected may be used to determine all or part of a sequence which is complementary to the receptor.

Use of the invention herein is illustrated primarily with reference to screening for biological activity. The invention will, however, find many other uses. For example, the invention may be used in information storage (e.g., on optical disks), production of molecular electronic devices, production of stationary phases in separation sciences, production of dyes and brightening agents, photography, and in immobilization of cells, proteins, lectins, nucleic acids, polysaccharides and the like in patterns on a surface via molecular recognition of specific polymer sequences. By synthesizing the same compound in adjacent, progressively differing concentrations, a gradient will be established to control chemotaxis or to develop diagnostic dipsticks which, for example, titrate an antibody against an increasing amount of antigen. By synthesizing several catalyst molecules in close proximity, more efficient multistep conversions may be achieved by "coordinate immobilization." Coordinate immobilization also may be used for electron transfer systems, as well as to provide both structural integrity and other desirable properties to materials such as lubrication, wetting, etc.

According to alternative embodiments, molecular biodistribution or pharmacokinetic properties may be examined. For example, to assess resistance to

intestinal or serum proteases, polymers may be capped with a fluorescent tag and exposed to biological fluids of interest.

III. Polymer Synthesis

Fig. 1 illustrates one embodiment of the invention disclosed herein in which a substrate 2 is shown in cross-section. Essentially, any conceivable substrate may be employed in the invention. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface is also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SiN_4, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoro-ethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or single-crystal silicon with surface relief features of less than 10 Å.

According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa

structures, or the like, the synthesis regions may be more closely placed within the focus point of impinging light, be provided with reflective "mirror" structures for maximization of light collection from fluorescent sources, or the like.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In some embodiments the surface may provide for the use of caged binding members which are attached firmly to the surface of the substrate in accord with the teaching of copending application Serial No. 404,920, previously incorporated herein by reference. Preferably, the surface will contain reactive groups, which could be carboxyl, amino, hydroxyl, or the like. Most preferably, the surface will be optically transparent and will have surface Si-OH functionalities, such as are found on silica surfaces.

The surface 4 of the substrate is preferably provided with a layer of linker molecules 6, although it will be understood that the linker molecules are not required elements of the invention. The linker molecules are preferably of sufficient length to permit polymers in a completed substrate to interact freely with molecules exposed to the substrate. The linker molecules should be 6-50 atoms long to provide sufficient exposure. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing 2-10 monomer units, diamines, diacids, amino acids, or combinations thereof. Other linker molecules may be used in light of this disclsoure.

According to alternative embodiments, the linker molecules are selected based upon their hydrophilic/hydrophobic properties to improve

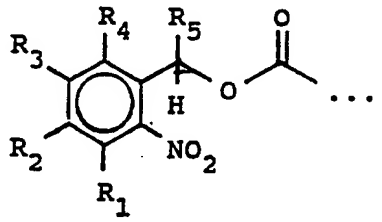
presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred so as to permit the receptor to more closely approach the synthesized polymer.

According to another alternative embodiment, linker molecules are also provided with a photocleavable group at an intermediate position. The photocleavable group is preferably cleavable at a wavelength different from the protective group. This enables removal of the various polymers following completion of the synthesis by way of exposure to the different wavelengths of light.

The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment via reactions of linker molecules bearing trichlorosilyl groups. The linker molecules may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized Langmuir Blodgett film. In alternative embodiments, the linker molecules are adsorbed to the surface of the substrate.

The linker molecules and monomers used herein are provided with a functional group to which is bound a protective group. Preferably, the protective group is on the distal or terminal end of the linker molecule opposite the substrate. The protective group may be either a negative protective group (i.e., the protective group renders the linker molecules less reactive with a monomer upon exposure) or a positive protective group (i.e., the protective group renders the linker molecules more reactive with a monomer upon exposure). In the case of negative protective groups an additional step of reactivation will be required. In some embodiments, this will be done by heating.

The protective group on the linker molecules may be selected from a wide variety of positive light-reactive groups preferably including nitro aromatic compounds such as o-nitrobenzyl derivatives or benzylsulfonyl. In a preferred embodiment, 6-nitroveratryloxycarbonyl (NVOC), 2-nitrobenzyloxycarbonyl (NBOC) or α,α-dimethyl-dimethoxybenzyloxycarbonyl (DDZ) is used. In one embodiment, a nitro aromatic compound containing a benzylic hydrogen ortho to the nitro group is used, i.e., a chemical of the form:



where R_1 is alkoxy, alkyl, halo, aryl, alkenyl, or hydrogen; R_2 is alkoxy, alkyl, halo, aryl, nitro, or hydrogen; R_3 is alkoxy, alkyl, halo, nitro, aryl, or hydrogen; R_4 is alkoxy, alkyl, hydrogen, aryl, halo, or nitro; and R_5 is alkyl, alkynyl, cyano, alkoxy, hydrogen, halo, aryl, or alkenyl. Other materials which may be used include o-hydroxy-α-methyl cinnamoyl derivatives. Photoremovable protective groups are described in, for example, Patchornik, J. Am. Chem. Soc. (1970) 92:6333 and Amit et al., J. Org. Chem. (1974) 39:192, both of which are incorporated herein by reference.

In an alternative embodiment the positive reactive group is activated for reaction with reagents in solution. For example, a 5-bromo-7-nitro indoline group, when bound to a carbonyl, undergoes reaction upon exposure to light at 420 nm.

In a second alternative embodiment, the reactive group on the linker molecule is selected from a wide variety of negative light-reactive groups including a cinammate group.

Alternatively, the reactive group is activated or deactivated by electron beam lithography, x-ray lithography, or any other radiation. Suitable reactive groups for electron beam lithography include sulfonyl. Other methods may be used including, for example, exposure to a current source. Other reactive groups and methods of activation may be used in light of this disclosure.

As shown in Fig. 1, the linking molecules are preferably exposed to, for example, light through a suitable mask 8 using photolithographic techniques of the type known in the semiconductor industry and described in, for example, Sze, VLSI Technology, McGraw-Hill (1983), and Mead et al., Introduction to VLSI Systems, Addison-Wesley (1980), which are incorporated herein by reference for all purposes. The light may be directed at either the surface containing the protective groups or at the back of the substrate, so long as the substrate is transparent to the wavelength of light needed for removal of the protective groups. In the embodiment shown in Fig. 1, light is directed at the surface of the substrate containing the protective groups. Fig. 1 illustrates the use of such masking techniques as they are applied to a positive reactive group so as to activate linking molecules and expose functional groups in areas 10a and 10b.

The mask 8 is in one embodiment a transparent support material selectively coated with a layer of opaque material. Portions of the opaque material are removed, leaving opaque material in the precise pattern desired on the substrate surface. The mask is brought into close proximity with, imaged on, or brought directly into contact with the substrate surface as shown in Fig. 1. "Openings" in the mask correspond to locations on the substrate where it is desired to remove photoremovable protective groups from the substrate. Alignment may be performed using conventional alignment

techniques in which alignment marks (not shown) are used to accurately overlay successive masks with previous patterning steps, or more sophisticated techniques may be used. For example, interferometric techniques such as the one described in Flanders et al., "A New Interferometric Alignment Technique," App. Phys. Lett. (1977) 31:426-428, which is incorporated herein by reference, may be used.

To enhance contrast of light applied to the substrate, it is desirable to provide contrast enhancement materials between the mask and the substrate according to some embodiments. This contrast enhancement layer may comprise a molecule which is decomposed by light such as quinone diazid or a material which is transiently bleached at the wavelength of interest. Transient bleaching of materials will allow greater penetration where light is applied, thereby enhancing contrast. Alternatively, contrast enhancement may be provided by way of a cladded fiber optic bundle.

The light may be from a conventional incandescent source, a laser, a laser diode, or the like. If non-collimated sources of light are used it may be desirable to provide a thick- or multi-layered mask to prevent spreading of the light onto the substrate. It may, further, be desirable in some embodiments to utilize groups which are sensitive to different wavelengths to control synthesis. For example, by using groups which are sensitive to different wavelengths, it is possible to select branch positions in the synthesis of a polymer or eliminate certain masking steps. Several reactive groups along with their corresponding wavelengths for deprotection are provided in Table 1.

Table 1

Group	Approximate Deprotection Wavelength
Nitroveratryloxy carbonyl (NVOC)	UV (300-400 nm)
Nitrobenzyloxy carbonyl (NBOC)	UV (300-350 nm)
Dimethyl dimethoxybenzyloxy carbonyl	UV (280-300 nm)
5-Bromo-7-nitroindolinyl	UV (420 nm)
o-Hydroxy-α-methyl cinnamoyl	UV (300-350 nm)
2-Oxymethylene anthraquinone	UV (350 nm)

While the invention is illustrated primarily herein by way of the use of a mask to illuminate selected regions the substrate, other techniques may also be used. For example, the substrate may be translated under a modulated laser or diode light source. Such techniques are discussed in, for example, U.S. Patent No. 4,719,615 (Feyrer et al.), which is incorporated herein by reference. In alternative embodiments a laser galvanometric scanner is utilized. In other embodiments, the synthesis may take place on or in contact with a conventional liquid crystal (referred to herein as a "light valve") or fiber optic light sources. By appropriately modulating liquid crystals, light may be selectively controlled so as to permit light to contact selected regions of the substrate. Alternatively, synthesis may take place on the end of a series of optical fibers to which light is selectively applied. Other means of controlling the location of light exposure will be apparent to those of skill in the art.

The substrate may be irradiated either in contact or not in contact with a solution (not shown) and is, preferably, irradiated in contact with a solution. The solution contains reagents to prevent the by-products formed by irradiation from interfering with synthesis of the polymer according to some embodiments. Such by-products might include, for example, carbon

dioxide, nitrosocarbonyl compounds, styrene derivatives, indole derivatives, and products of their photochemical reactions. Alternatively, the solution may contain reagents used to match the index of refraction of the substrate. Reagents added to the solution may further include, for example, acidic or basic buffers, thiols, substituted hydrazines and hydroxylamines, reducing agents (e.g., NADH) or reagents known to react with a given functional group (e.g., aryl nitroso + glyoxylic acid → aryl formhydroxamate + CO_2).

Either concurrently with or after the irradiation step, the linker molecules are washed or otherwise contacted with a first monomer, illustrated by "A" in regions 12a and 12b in Fig. 2. The first monomer reacts with the activated functional groups of the linkage molecules which have been exposed to light. The first monomer, which is preferably an amino acid, is also provided with a photoprotective group. The photoprotective group on the monomer may be the same as or different than the protective group used in the linkage molecules, and may be selected from any of the above-described protective groups. In one embodiment, the protective groups for the A monomer is selected from the group NBOC and NVOC.

As shown in Fig. 3, the process of irradiating is thereafter repeated, with a mask repositioned so as to remove linkage protective groups and expose functional groups in regions 14a and 14b which are illustrated as being regions which were protected in the previous masking step. As an alternative to repositioning of the first mask, in many embodiments a second mask will be utilized. In other alternative embodiments, some steps may provide for illuminating a common region in successive steps. As shown in Fig. 3, it may be desirable to provide separation between irradiated regions. For example, separation of about 1-5 μm may be appropriate to account for alignment tolerances.

As shown in Fig. 4, the substrate is then exposed to a second protected monomer "B," producing B regions 16a and 16b. Thereafter, the substrate is again masked so as to remove the protective groups and expose reactive groups on A region 12a and B region 16b. The substrate is again exposed to monomer B, resulting in the formation of the structure shown in Fig. 6. The dimers B-A and B-B have been produced on the substrate.

A subsequent series of masking and contacting steps similar to those described above with A (not shown) provides the structure shown in Fig. 7. The process provides all possible dimers of B and A, i.e., B-A, A-B, A-A, and B-B.

The substrate, the area of synthesis, and the area for synthesis of each individual polymer could be of any size or shape. For example, squares, ellipsoids, rectangles, triangles, circles, or portions thereof, along with irregular geometric shapes, may be utilized. Duplicate synthesis areas may also be applied to a single substrate for purposes of redundancy.

In one embodiment the regions 12 and 16 on the substrate will have a surface area of between about 1 cm^2 and 10^{-10} cm^2. In some embodiments the regions 12 and 16 have areas of less than about 10^{-1} cm^2, 10^{-2} cm^2, 10^{-3} cm^2, 10^{-4} cm^2, 10^{-5} cm^2, 10^{-6} cm^2, 10^{-7} cm^2, 10^{-8} cm^2, or 10^{-10} cm^2. In a preferred embodiment, the regions 12 and 16 are between about 10x10 μm and 500x500 μm.

In some embodiments a single substrate supports more than about 10 different monomer sequences and perferably more than about 100 different monomer sequences, although in some embodiments more than about 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 different sequences are provided on a substrate. Of course, within a region of the substrate in which a monomer sequence is synthesized, it is preferred that the monomer sequence be substantially pure. In some embodiments, regions of the substrate contain polymer sequences which are at

least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% pure.

According to some embodiments, several sequences are intentionally provided within a single region so as to provide an initial screening for biological activity, after which materials within regions exhibiting significant binding are further evaluated.

IV. Details of One Embodiment of a Reactor System

Fig. 8A schematically illustrates a preferred embodiment of a reactor system 100 for synthesizing polymers on the prepared substrate in accordance with one aspect of the invention. The reactor system includes a body 102 with a cavity 104 on a surface thereof. In preferred embodiments the cavity 104 is between about 50 and 1000 μm deep with a depth of about 500 μm preferred.

The bottom of the cavity is preferably provided with an array of ridges 106 which extend both into the plane of the Figure and parallel to the plane of the Figure. The ridges are preferably about 50 to 200 μm deep and spaced at about 2 to 3mm. The purpose of the ridges is to generate turbulent flow for better mixing. The bottom surface of the cavity is preferably light absorbing so as to prevent reflection of impinging light.

A substrate 112 is mounted above the cavity 104. The substrate is provided along its bottom surface 114 with a photoremovable protective group such as NVOC with or without an intervening linker molecule. The substrate is preferably transparent to a wide spectrum of light, but in some embodiments is transparent only at a wavelength at which the protective group may be removed (such as UV in the case of NVOC). The substrate in some embodiments is a conventional microscope glass slide or cover slip. The substrate is preferably as thin as possible, while still providing adequate physical support. Preferably, the substrate is less than about

1 mm thick, more preferably less than 0.5 mm thick, more preferably less than 0.1 mm thick, and most preferably less than 0.05 mm thick. In alternative preferred embodiments, the substrate is quartz or silicon.

The substrate and the body serve to seal the cavity except for an inlet port 108 and an outlet port 110. The body and the substrate may be mated for sealing in some embodiments with one or more gaskets. According to a preferred embodiment, the body is provided with two concentric gaskets and the intervening space is held at vacuum to ensure mating of the substrate to the gaskets.

Fluid is pumped through the inlet port into the cavity by way of a pump 116 which may be, for example, a model no. B-120-S made by Eldex Laboratories. Selected fluids are circulated into the cavity by the pump, through the cavity, and out the outlet for recirculation or disposal. The reactor may be subjected to ultrasonic radiation and/or heated to aid in agitation in some embodiments.

Above the substrate 112, a lens 120 is provided which may be, for example, a 2" 100mm focal length fused silica lens. For the sake of a compact system, a reflective mirror 122 may be provided for directing light from a light source 124 onto the substrate. Light source 124 may be, for example, a Xe(Hg) light source manufactured by Oriel and having model no. 66024. A second lens 126 may be provided for the purpose of projecting a mask image onto the substrate in combination with lens 112. This form of lithography is referred to herein as projection printing. As will be apparent from this disclosure, proximity printing and the like may also be used according to some embodiments.

Light from the light source is permitted to reach only selected locations on the substrate as a result of mask 128. Mask 128 may be, for example, a glass slide having etched chrome thereon. The mask 128 in one embodiment is provided with a grid of transparent

locations and opaque locations. Such masks may be manufactured by, for example, Photo Sciences, Inc. Light passes freely through the transparent regions of the mask, but is reflected from or absorbed by other regions. Therefore, only selected regions of the substrate are exposed to light.

As discussed above, light valves (LCD's) may be used as an alternative to conventional masks to selectively expose regions of the substrate. Alternatively, fiber optic faceplates such as those available from Schott Glass, Inc, may be used for the purpose of contrast enhancement of the mask or as the sole means of restricting the region to which light is applied. Such faceplates would be placed directly above or on the substrate in the reactor shown in Fig. 8A. In still further embodiments, flys-eye lenses, tapered fiber optic faceplates, or the like, may be used for contrast enhancement.

In order to provide for illumination of regions smaller than a wavelength of light, more elaborate techniques may be utilized. For example, according to one preferred embodiment, light is directed at the substrate by way of molecular microcrystals on the tip of, for example, micropipettes. Such devices are disclosed in Lieberman et al., "A Light Source Smaller Than the Optical Wavelength," Science (1990) 247:59-61, which is incorporated herein by reference for all purposes.

In operation, the substrate is placed on the cavity and sealed thereto. All operations in the process of preparing the substrate are carried out in a room lit primarily or entirely by light of a wavelength outside of the light range at which the protective group is removed. For example, in the case of NVOC, the room should be lit with a conventional dark room light which provides little or no UV light. All operations are preferably conducted at about room temperature.

A first, deprotection fluid (without a monomer) is circulated through the cavity. The solution preferably is of 5 mM sulfuric acid in dioxane solution which serves to keep exposed amino groups protonated and decreases their reactivity with photolysis by-products. Absorptive materials such as N,N-diethylamino 2,4-dinitrobenzene, for example, may be included in the deprotection fluid which serves to absorb light and prevent reflection and unwanted photolysis.

The slide is, thereafter, positioned in a light raypath from the mask such that first locations on the substrate are illuminated and, therefore, deprotected. In preferred embodiments the substrate is illuminated for between about 1 and 15 minutes with a preferred illumination time of about 10 minutes at 10-20 mW/cm^2 with 365 nm light. The slides are neutralized (i.e., brought to a pH of about 7) after photolysis with, for example, a solution of di-isopropylethylamine (DIEA) in methylene chloride for about 5 minutes.

The first monomer is then placed at the first locations on the substrate. After irradiation, the slide is removed, treated in bulk, and then reinstalled in the flow cell. Alternatively, a fluid containing the first monomer, preferably also protected by a protective group, is circulated through the cavity by way of pump 116. If, for example, it is desired to attach the amino acid Y to the substrate at the first locations, the amino acid Y (bearing a protective group on its α-nitrogen), along with reagents used to render the monomer reactive, and/or a carrier, is circulated from a storage container 118, through the pump, through the cavity, and back to the inlet of the pump.

The monomer carrier solution is, in a preferred embodiment, formed by mixing of a first solution (referred to herein as solution "A") and a second solution (referred to herein as solution "B"). Table 2

provides an illustration of a mixture which may be used for solution A.

Table 2
Representative Monomer Carrier Solution "A"

100 mg NVOC amino protected amino acid
37 mg HOBT (1-Hydroxybenzotriazole)
250 µl DMF (Dimethylformamide)
86 µl DIEA (Diisopropylethylamine)

The composition of solution B is illustrated in Table 3. Solutions A and B are mixed and allowed to react at room temperature for about 8 minutes, then diluted with 2 ml of DMF, and 500 µl are applied to the surface of the slide or the solution is circulated through the reactor system and allowed to react for about 2 hours at room temperature. The slide is then washed with DMF, methylene chloride and ethanol.

Table 3
Representative Monomer Carrier Solution "B"

250 µl DMF
111 mg BOP (Benzotriazolyl-n-oxy-tris(dimethylamino) phosphoniumhexafluorophosphate)

As the solution containing the monomer to be attached is circulated through the cavity, the amino acid or other monomer will react at its carboxy terminus with amino groups on the regions of the substrate which have been deprotected. Of course, while the invention is illustrated by way of circulation of the monomer through the cavity, the invention could be practiced by way of

removing the slide from the reactor and submersing it in an appropriate monomer solution.

After addition of the first monomer, the solution containing the first amino acid is then purged from the system. After circulation of a sufficient amount of the DMF/methylene chloride such that removal of the amino acid can be assured (e.g., about 50x times the volume of the cavity and carrier lines), the mask or substrate is repositioned, or a new mask is utilized such that second regions on the substrate will be exposed to light and the light 124 is engaged for a second exposure. This will deprotect second regions on the substrate and the process is repeated until the desired polymer sequences have been synthesized.

The entire derivatized substrate is then exposed to a receptor of interest, preferably labeled with, for example, a fluorescent marker, by circulation of a solution or suspension of the receptor through the cavity or by contacting the surface of the slide in bulk. The receptor will preferentially bind to certain regions of the substrate which contain complementary sequences.

Antibodies are typically suspended in what is commonly referred to as "supercocktail," which may be, for example, a solution of about 1% BSA (bovine serum albumin), 0.5% Tween in PBS (phosphate buffered saline) buffer. The antibodies are diluted into the supercocktail buffer to a final concentration of, for example, about 0.1 to 4 μg/ml.

Fig. 8B illustrates an alternative preferred embodiment of the reactor shown in Fig. 8A. According to this embodiment, the mask 128 is placed directly in contact with the substrate. Preferably, the etched portion of the mask is placed face down so as to reduce the effects of light dispersion. According to this embodiment, the imaging lenses 120 and 126 are not necessary because the mask is brought into close proximity with the substrate.

For purposes of increasing the signal-to-noise ratio of the technique, some embodiments of the invention provide for exposure of the substrate to a first labeled or unlabeled receptor followed by exposure of a labeled, second receptor (e.g., an antibody) which binds at multiple sites on the first receptor. If, for example, the first receptor is an antibody derived from a first species of an animal, the second receptor is an antibody derived from a second species directed to epitopes associated with the first species. In the case of a mouse antibody, for example, fluorescently labeled goat antibody or antiserum which is antimouse may be used to bind at multiple sites on the mouse antibody, providing several times the fluorescence compared to the attachment of a single mouse antibody at each binding site. This process may be repeated again with additional antibodies (e.g., goat-mouse-goat, etc.) for further signal amplification.

In preferred embodiments an ordered sequence of masks is utilized. In some embodiments it is possible to use as few as a single mask to synthesize all of the possible polymers of a given monomer set.

If, for example, it is desired to synthesize all 16 dinucleotides from four bases, a 1 cm square synthesis region is divided conceptually into 16 boxes, each 0.25 cm wide. Denote the four monomer units by A, B, C, and D. The first reactions are carried out in four vertical columns, each 0.25 cm wide. The first mask exposes the left-most column of boxes, where A is coupled. The second mask exposes the next column, where B is coupled; followed by a third mask, for the C column; and a final mask that exposes the right-most column, for D. The first, second, third, and fourth masks may be a single mask translated to different locations.

The process is repeated in the horizontal direction for the second unit of the dimer. This time, the masks allow exposure of horizontal rows, again 0.25 cm wide. A, B, C, and D are sequentially coupled using masks that expose horizontal fourths of the reaction area. The resulting substrate contains all 16 dinucleotides of four bases.

The eight masks used to synthesize the dinucleotide are related to one another by translation or rotation. In fact, one mask can be used in all eight steps if it is suitably rotated and translated. For example, in the example above, a mask with a single transparent region could be sequentially used to expose each of the vertical columns, translated 90°, and then sequentially used to allow exposure of the horizontal rows.

Tables 4 and 5 provide a simple computer program in Quick Basic for planning a masking program and a sample output, respectively, for the synthesis of a polymer chain of three monomers ("residues") having three different monomers in the first level, four different monomers in the second level, and five different monomers in the third level in a striped pattern. The output of the program is the number of cells, the number of "stripes" (light regions) on each mask, and the amount of translation required for each exposure of the mask.

Table 4

Mask Strategy Program

```
DEFINT A-Z
DIM b(20), w(20), 1(500)
F$ = "LPT1:"
OPEN f$ FOR OUTPUT AS #1

jmax = 3        'Number of residues
b(1) = 3: b(2) = 4: b(3) = 5     'Number of building blocks for res 1,2,3
g = 1: lmax(1) = 1

FOR j = 1 TO jmax: g= g * b(j): NEXT j

w(0) = 0: w(1) = g / b(1)

PRINT #1, "MASK2.BAS ", DATE$, TIME$: PRINT #1,
PRINT #1, USING "Number of residues=##"; jmax
FOR j = 1 TO jmax
PRINT #1, USING "    Residue ##      ## building blocks"; j; b(j)
NEXT j
PRINT #1, "
PRINT #1, USING "Number of cells=####"; g: PRINT #1,

FOR j = 2 TO jmax
lmax(j) = lmax(j - 1) * b(j - 1)
w(j) = w(j - 1) / b(j)
NEXT j

FOR j = 1 TO jmax
PRINT #1, USING "Mask for residue ##"; j: PRINT #1,
PRINT #1, USING "   Number of stripes=###"; lmax(j)
PRINT #1, USING "   Width of each stripe=###"; w(j)
FOR 1 = 1 TO lmax(j)
a = 1 + (1 - 1) * w(j - 1)
ae = a + w(j) - 1
PRINT #1, USING "   Stripe ## begins at location ### and ends at ###"; 1; a; ae
NEXT 1
PRINT #1,
PRINT #1, USING "   For each of ## building blocks, translate mask by ##
cell(s)"; b(j); w(j),
PRINT #1, : PRINT #1, : PRINT #1,
NEXT j
```

Table 5

Masking Strategy Output

```
Number of residues= 3
     Residue  1       3 building blocks
     Residue  2       4 building blocks
     Residue  3       5 building blocks

Number of cells=  60

Mask for residue  1

   Number of stripes=  1
   Width of each stripe= 20
   Stripe  1 begins at location   1 and ends at  20

   For each of  3 building blocks, translate mask by 20 cell(s)


Mask for residue  2

   Number of stripes=  3
   Width of each stripe= 5
   Stripe  1 begins at location   1 and ends at   5
   Stripe  2 begins at location  21 and ends at  25
   Stripe  3 begins at location  41 and ends at  45

   For each of  4 building blocks, translate mask by  5 cell(s)


Mask for residue  3

   Number of stripes= 12
   Width of each stripe= 1
   Stripe  1 begins at location   1 and ends at   1
   Stripe  2 begins at location   6 and ends at   6
   Stripe  3 begins at location  11 and ends at  11
   Stripe  4 begins at location  16 and ends at  16
   Stripe  5 begins at location  21 and ends at  21
   Stripe  6 begins at location  26 and ends at  26
   Stripe  7 begins at location  31 and ends at  31
   Stripe  8 begins at location  36 and ends at  36
   Stripe  9 begins at location  41 and ends at  41
   Stripe 10 begins at location  46 and ends at  46
   Stripe 11 begins at location  51 and ends at  51
   Stripe 12 begins at location  56 and ends at  56

   For each of  5 building blocks, translate mask by  1 cell(s)
```

V. Details of One Embodiment of A Fluorescent Detection Device

Fig. 9 illustrates a fluorescent detection device for detecting fluorescently labeled receptors on a substrate. A substrate 112 is placed on an x/y translation table 202. In a preferred embodiment the x/y translation table is a model no. PM500-A1 manufactured by Newport Corporation. The x/y translation table is connected to and controlled by an appropriately programmed digital computer 204 which may be, for example, an appropriately programmed IBM PC/AT or AT compatible computer. Of course, other computer systems, special purpose hardware, or the like could readily be substituted for the AT computer used herein for illustration. Computer software for the translation and data collection functions described herein can be provided based on commercially available software including, for example, "Lab Windows" licensed by National Instruments, which is incorporated herein by reference for all purposes.

The substrate and x/y translation table are placed under a microscope 206 which includes one or more objectives 208. Light (about 488 nm) from a laser 210, which in some embodiments is a model no. 2020-05 argon ion laser manufactured by Spectraphysics, is directed at the substrate by a dichroic mirror 207 which passes greater than about 520 nm light but reflects 488 nm light. Dichroic mirror 207 may be, for example, a model no. FT510 manufactured by Carl Zeiss. Light reflected from the mirror then enters the microscope 206 which may be, for example, a model no. Axioscop 20 manufactured by Carl Zeiss. Fluorescein-marked materials on the substrate will fluoresce >488 nm light, and the fluoresced light will be collected by the microscope and passed through the mirror. The fluorescent light from the substrate is then directed through a wavelength filter 209 and, thereafter through an aperture plate 211.

Wavelength filter 209 may be, for example, a model no. OG530 manufactured by Melles Griot and aperture plate 211 may be, for example, a model no. 477352/477380 manufactured by Carl Zeiss.

The fluoresced light then enters a photomultiplier tube 212 which in some embodiments is a model no. R943-02 manufactured by Hamamatsu, the signal is amplified in preamplifier 214 and photons are counted by photon counter 216. The number of photons is recorded as a function of the location in the computer 204. Pre-Amp 214 may be, for example, a model no. SR440 manufactured by Stanford Research Systems and photon counter 216 may be a model no. SR400 manufactured by Stanford Research Systems. The substrate is then moved to a subsequent location and the process is repeated. In preferred embodiments the data are acquired every 1 to 100 μm with a data collection diameter of about 0.8 to 10 μm preferred. In embodiments with sufficiently high fluorescence, a CCD detector with broadfield illumination is utilized.

By counting the number of photons generated in a given area in response to the laser, it is possible to determine where fluorescent marked molecules are located on the substrate. Consequently, for a slide which has a matrix of polypeptides, for example, synthesized on the surface thereof, it is possible to determine which of the polypeptides is complementary to a fluorescently marked receptor.

According to preferred embodiments, the intensity and duration of the light applied to the substrate is controlled by varying the laser power and scan stage rate for improved signal-to-noise ratio by maximizing fluorescence emission and minimizing background noise.

While the detection apparatus has been illustrated primarily herein with regard to the detection of marked receptors, the invention will find application

in other areas. For example, the detection apparatus disclosed herein could be used in the fields of catalysis, DNA or protein gel scanning, and the like.

VI. Determination of Relative Binding Strength of Receptors

The signal-to-noise ratio of the present invention is sufficiently high that not only can the presence or absence of a receptor on a ligand be detected, but also the relative binding affinity of receptors to a variety of sequences can be determined.

In practice it is found that a receptor will bind to several peptide sequences in an array, but will bind much more strongly to some sequences than others. Strong binding affinity will be evidenced herein by a strong fluorescent or radiographic signal since many receptor molecules will bind in a region of a strongly bound ligand. Conversely, a weak binding affinity will be evidenced by a weak fluorescent or radiographic signal due to the relatively small number of receptor molecules which bind in a particular region of a substrate having a ligand with a weak binding affinity for the receptor. Consequently, it becomes possible to determine relative binding avidity (or affinity in the case of univalent interactions) of a ligand herein by way of the intensity of a fluorescent or radiographic signal in a region containing that ligand.

Semiquantitative data on affinities might also be obtained by varying washing conditions and concentrations of the receptor. This would be done by comparison to known ligand receptor pairs, for example.

VII. Examples

The following examples are provided to illustrate the efficacy of the inventions herein. All operations were conducted at about ambient temperatures and pressures unless indicated to the contrary.

A. Slide Preparation

Before attachment of reactive groups it is preferred to clean the substrate which is, in a preferred embodiment a glass substrate such as a microscope slide or cover slip. According to one embodiment the slide is soaked in an alkaline bath consisting of, for example, 1 liter of 95% ethanol with 120 ml of water and 120 grams of sodium hydroxide for 12 hours. The slides are then washed under running water and allowed to air dry, and rinsed once with a solution of 95% ethanol.

The slides are then aminated with, for example, aminopropyltriethoxysilane for the purpose of attaching amino groups to the glass surface on linker molecules, although any omega functionalized silane could also be used for this purpose. In one embodiment 0.1% aminopropyltriethoxysilane is utilized, although solutions with concentrations from 10^{-7}% to 10% may be used, with about 10^{-3}% to 2% preferred. A 0.1% mixture is prepared by adding to 100 ml of a 95% ethanol/5% water mixture, 100 microliters (µl) of aminopropyltriethoxysilane. The mixture is agitated at about ambient temperature on a rotary shaker for about 5 minutes. 500 µl of this mixture is then applied to the surface of one side of each cleaned slide. After 4 minutes, the slides are decanted of this solution and rinsed three times by dipping in, for example, 100% ethanol.

After the plates dry, they are placed in a 110-120°C vacuum oven for about 20 minutes, and then allowed to cure at room temperature for about 12 hours in an argon environment. The slides are then dipped into DMF (dimethylformamide) solution, followed by a thorough washing with methylene chloride.

The aminated surface of the slide is then exposed to about 500 µl of, for example, a 30 millimolar (mM) solution of NVOC-GABA (gamma amino butyric acid) NHS (N-hydroxysuccinimide) in DMF for attachment of a NVOC-GABA to each of the amino groups.

The surface is washed with, for example, DMF, methylene chloride, and ethanol.

Any unreacted aminopropyl silane on the surface--that is, those amino groups which have not had the NVOC-GABA attached--are now capped with acetyl groups (to prevent further reaction) by exposure to a 1:3 mixture of acetic anhydride in pyridine for 1 hour. Other materials which may perform this residual capping function include trifluoroacetic anhydride, formicacetic anhydride, or other reactive acylating agents. Finally, the slides are washed again with DMF, methylene chloride, and ethanol.

B. Synthesis of Eight Trimers of "A" and "B"

Fig. 10 illustrates a possible synthesis of the eight trimers of the two-monomer set: gly, phe (represented by "A" and "B," respectively). A glass slide bearing silane groups terminating in 6-nitro-veratryloxycarboxamide (NVOC-NH) residues is prepared as a substrate. Active esters (pentafluorophenyl, OBt, etc.) of gly and phe protected at the amino group with NVOC are prepared as reagents. While not pertinent to this example, if side chain protecting groups are required for the monomer set, these must not be photoreactive at the wavelength of light used to protect the primary chain.

For a monomer set of size n, n x ℓ cycles are required to synthesize all possible sequences of length ℓ. A cycle consists of:

1. Irradiation through an appropriate mask to expose the amino groups at the sites where the next residue is to be added, with appropriate washes to remove the by-products of the deprotection.
2. Addition of a single activated and protected (with the same photochemically-removable group) monomer, which will react

only at the sites addressed in step 1, with appropriate washes to remove the excess reagent from the surface.

The above cycle is repeated for each member of the monomer set until each location on the surface has been extended by one residue in one embodiment. In other embodiments, several residues are sequentially added at one location before moving on to the next location. Cycle times will generally be limited by the coupling reaction rate, now as short as 20 min in automated peptide synthesizers. This step is optionally followed by addition of a protecting group to stabilize the array for later testing. For some types of polymers (e.g., peptides), a final deprotection of the entire surface (removal of photoprotective side chain groups) may be required.

More particularly, as shown in Fig. 10A, the glass 20 is provided with regions 22, 24, 26, 28, 30, 32, 34, and 36. Regions 30, 32, 34, and 36 are masked, as shown in Fig. 10B and the glass is irradiated and exposed to a reagent containg "A" (e.g., gly), with the resulting structure shown in Fig. 10C. Thereafter, regions 22, 24, 26, and 28 are masked, the glass is irradiated (as shown in Fig. 10D) and exposed to a reagent containing "B" (e.g., phe), with the resulting structure shown in Fig. 10E. The process proceeds, consecutively masking and exposing the sections as shown until the structure shown in Fig. 10M is obtained. The glass is irradiated and the terminal groups are, optionally, capped by acetylation. As shown, all possible trimers of gly/phe are obtained.

In this example, no side chain protective group removal is necessary. If it is desired, side chain deprotection may be accomplished by treatment with ethanedithiol and trifluoroacetic acid.

In general, the number of steps needed to obtain a particular polymer chain is defined by:

$$n \times \ell \quad (1)$$

where:

n = the number of monomers in the basis set of monomers, and

ℓ = the number of monomer units in a polymer chain.

Conversely, the synthesized number of sequences of length ℓ will be:

$$n^{\ell}. \quad (2)$$

Of course, greater diversity is obtained by using masking strategies which will also include the synthesis of polymers having a length of less than ℓ. If, in the extreme case, all polymers having a length less than or equal to ℓ are synthesized, the number of polymers synthesized will be:

$$n^{\ell} + n^{\ell-1} + \ldots + n^{1}. \quad (3)$$

The maximum number of lithographic steps needed will generally be n for each "layer" of monomers, i.e., the total number of masks (and, therefore, the number of lithographic steps) needed will be $n \times \ell$. The size of the transparent mask regions will vary in accordance with the area of the substrate available for synthesis and the number of sequences to be formed. In general, the size of the synthesis areas will be:

$$\text{size of synthesis areas} = (A)/(S)$$

where:

A is the total area available for synthesis; and

S is the number of sequences desired in the area.

It will be appreciated by those of skill in the art that the above method could readily be used to simultaneously produce thousands or millions of oligomers on a substrate using the photolithographic techniques disclosed herein. Consequently, the method results in the ability to practically test large numbers of, for example, di, tri, tetra, penta, hexa, hepta, octapeptides, dodecapeptides, or larger polypeptides (or correspondingly, polynucleotides).

The above example has illustrated the method by way of a manual example. It will of course be appreciated that automated or semi-automated methods could be used. The substrate would be mounted in a flow cell for automated addition and removal of reagents, to minimize the volume of reagents needed, and to more carefully control reaction conditions. Successive masks could be applied manually or automatically.

C. Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group

In synthesizing the dimer of an aminopropyl group and a fluorescent group, a functionalized durapore membrane was used as a substrate. The durapore membrane was a polyvinylidine difluoride with aminopropyl groups. The aminopropyl groups were protected with the DDZ group by reaction of the carbonyl chloride with the amino groups, a reaction readily known to those of skill in the art. The surface bearing these groups was placed in a solution of THF and contacted with a mask bearing a checkerboard pattern of 1 mm opaque and transparent regions. The mask was exposed to ultraviolet light having a wavelength down to at least about 280 nm for about 5 minutes at ambient temperature, although a wide range of exposure times and temperatures may be

appropriate in various embodiments of the invention. For example, in one embodiment, an exposure time of between about 1 and 5000 seconds may be used at process temperatures of between -70 and +50°C.

In one preferred embodiment, exposure times of between about 1 and 500 seconds at about ambient pressure are used. In some preferred embodiments, pressure above ambient is used to prevent evaporation.

The surface of the membrane was then washed for about 1 hour with a fluorescent label which included an active ester bound to a chelate of a lanthanide. Wash times will vary over a wide range of values from about a few minutes to a few hours. These materials fluoresce in the red and the green visible region. After the reaction with the active ester in the fluorophore was complete, the locations in which the fluorophore was bound could be visualized by exposing them to ultraviolet light and observing the red and the green fluorescence. It was observed that the derivatized regions of the substrate closely corresponded to the original pattern of the mask.

D. Demonstration of Signal Capability

Signal detection capability was demonstrated using a low-level standard fluorescent bead kit manufactured by Flow Cytometry Standarda and having model no. 824. This kit includes 5.8 μm diameter beads, each impregnated with a known number of fluorescein molecules.

One of the beads was placed in the illumination field on the scan stage as shown in Fig. 9 in a field of a laser spot which was initially shuttered. After being positioned in the illumination field, the photon detection equipment was turned on. The laser beam was unblocked and it interacted with the particle bead, which then fluoresced. Fluorescence curves of beads impregnated with 7,000 and 29,000 fluorescein molecules, are shown in Figs. 11A and 11B, respectively. On each

curve, traces for beads without fluorescein molecules are also shown. These experiments were performed with 488 nm excitation, with 100 μW of laser power. The light was focused through a 40 power 0.75 NA objective.

The fluorescence intensity in all cases started off at a high value and then decreased exponentially. The fall-off in intensity is due to photobleaching of the fluorescein molecules. The traces of beads without fluorescein molecules are used for background subtraction. The difference in the initial exponential decay between labeled and nonlabeled beads is integrated to give the total number of photon counts, and this number is related to the number of molecules per bead. Therefore, it is possible to deduce the number of photons per fluorescein molecule that can be detected. For the curves illustrated in Fig. 11, this calculation indicates the radiation of about 40 to 50 photons per fluorescein molecule are detected.

E. Determination of the Number of Molecules Per Unit Area

Aminopropylated glass microscope slides prepared according to the methods discussed above were utilized in order to establish the density of labeling of the slides. The free amino termini of the slides were reacted with FITC (fluorescein isothiocyanate) which forms a covalent linkage with the amino group. The slide is then scanned to count the number of fluorescent photons generated in a region which, using the estimated 40-50 photons per fluorescent molecule, enables the calculation of the number of molecules which are on the surface per unit area.

A slide with aminopropyl silane on its surface was immersed in a 1 mM solution of FITC in DMF for 1 hour at about ambient temperature. After reaction, the slide was washed twice with DMF and then washed with ethanol, water, and then ethanol again. It was then

dried and stored in the dark until it was ready to be examined.

Through the use of curves similar to those shown in Fig. 11, and by integrating the fluorescent counts under the exponentially decaying signal, the number of free amino groups on the surface after derivitization was determined. It was determined that slides with labeling densities of 1 fluoroscein per $10^3 x 10^3$ to ~2x2 nm could be reproducibly made as the concentration of aminopropyltriethoxysilane varied from 10^{-5}% to 10^{-1}%.

F. Removal of NVOC and Attachment of A Fluorescent Marker

NVOC-GABA groups were attached as described above. The entire surface of one slide was exposed to light so as to expose a free amino group at the end of the gamma amino butyric acid. This slide, and a duplicate which was not exposed, were then exposed to fluorescein isothiocyanate (FITC).

Fig. 12A illustrates the slide which was not exposed to light, but which was exposed to FITC. The units of the x axis are time and the units of the y axis are counts. The trace contains a certain amount of background fluorescence. The duplicate slide was exposed to 350 nm broadband illumination for about 1 minute (12 mW/cm^2, ~350 nm illumination), washed and reacted with FITC. The fluorescence curves for this slide are shown in Fig. 12B. A large increase in the level of fluorescence is observed, which indicates photolysis has exposed a number of amino groups on the surface of the slides for attachment of a fluorescent marker.

G. Use of a Mask in Removal of NVOC

The next experiment was performed with a 0.1% aminopropylated slide. Light from a Hg-Xe arc lamp was imaged onto the substrate through a laser-ablated chrome-on-glass mask in direct contact with the substrate.

This slide was illuminated for approximately 5 minutes, with 12 mW of 350 nm broadband light and then reacted with the 1 mM FITC solution. It was put on the laser detection scanning stage and a graph was plotted as a two-dimensional representation of position color-coded for fluorescence intensity. The experiment was repeated a number of times through various masks. The fluorescence patterns for a 100x100 μm mask, a 50 μm mask, a 20 μm mask, and a 10 μm mask indicate that the mask pattern is distinct down to at least about 10 μm squares using this lithographic technique.

H. Attachment of YGGFL and Subsequent Exposure to Herz Antibody and Goat Antimouse

In order to establish that receptors to a particular polypeptide sequence would bind to a surface-bound peptide and be detected, Leu enkephalin was coupled to the surface and recognized by an antibody. A slide was derivatized with 0.1% amino propyl-triethoxysilane and protected with NVOC. A 500 μm checkerboard mask was used to expose the slide in a flow cell using backside contact printing. The Leu enkephalin sequence (H_2N-tyrosine,glycine,glycine,phenylalanine,leucine-CO_2H, otherwise referred to herein as YGGFL) was attached via its carboxy end to the exposed amino groups on the surface of the slide. The peptide was added in DMF solution with the BOP/HOBT/DIEA coupling reagents and recirculated through the flow cell for 2 hours at room temperature.

A first antibody, known as the Herz antibody, was applied to the surface of the slide for 45 minutes

at 2 μg/ml in a supercocktail (containing 1% BSA and 1% ovalbumin also in this case). A second antibody, goat anti-mouse fluorescein conjugate, was then added at 2 μg/ml in the supercocktail buffer, and allowed to incubate for 2 hours.

The results of this experiment were plotted as fluorescence intensity as a function of position. This image was taken at 10 μm steps and showed that not only can deprotection be carried out in a well defined pattern, but also that (1) the method provided for successful coupling of peptides to the surface of the substrate, (2) the surface of a bound peptide was available for binding with an antibody, and (3) that the detection apparatus capabilities were sufficient to detect binding of a receptor.

I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody

Monomer-by-monomer synthesis of YGGFL and GGFL in alternate squares was performed on a slide in a checkerboard pattern and the resulting slide was exposed to the Herz antibody. This experiment is illustrated in Figs. 13A and 13B.

In Fig. 13A, a slide is shown which is derivatized with the aminopropyl group, protected in this case with t-BOC (t-butoxycarbonyl). The slide was treated with TFA to remove the t-BOC protecting group. E-aminocaproic acid, which was t-BOC protected at its amino group, was then coupled onto the aminopropyl groups. The aminocaproic acid serves as a spacer between the aminopropyl group and the peptide to be synthesized. The amino end of the spacer was deprotected and coupled to NVOC-leucine. The entire slide was then illuminated with 12 mW of 325 nm broadband illumination. The slide was then coupled with NVOC-phenylalanine and washed. The entire slide was again illuminated, then coupled to NVOC-glycine and washed. The slide was again illuminated

and coupled to NVOC-glycine to form the sequence shown in the last portion of Fig. 13A.

As shown in Fig. 13B, alternating regions of the slide were then illuminated using a projection print using a 500x500 μm checkerboard mask; thus, the amino group of glycine was exposed only in the lighted areas. When the next coupling chemistry step was carried out, NVOC-tyrosine was added, and it coupled only at those spots which had received illumination. The entire slide was then illuminated to remove all the NVOC groups, leaving a checkerboard of YGGFL in the lighted areas and in the other areas, GGFL. The Herz antibody (which recognizes the YGGFL, but not GGFL) was then added, followed by goat anti-mouse fluorescein conjugate.

The resulting fluorescence scan showed dark areas containing the tetrapeptide GGFL, which is not recognized by the Herz antibody (and thus there is no binding of the goat anti-mouse antibody with fluorescein conjugate), and red areas in which YGGFL was present. The YGGFL pentapeptide is recognized by the Herz antibody and, therefore, there is antibody in the lighted regions for the fluorescein-conjugated goat anti-mouse to recognize.

Similar patterns for a 50 μm mask used in direct contact ("proximity print") with the substrate provided a pattern which was more distinct and the corners of the checkerboard pattern were touching as a result of the mask being placed in direct contact with the substrate (which reflects the increase in resolution using this technique).

J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL

A synthesis using a 50 μm checkerboard mask similar to that shown in Fig. 13 was conducted. However, P was added to the GGFL sites on the substrate through an additional coupling step. P was added by exposing protected GGFL to light through a mask, and subsequence

exposure to P in the manner set forth above. Therefore, half of the regions on the substrate contained YGGFL and the remaining half contained PGGFL.

The fluorescence plot for this experiment showed the regions are again readily discernable between those in which binding did and did not occur. This experiment demonstrated that antibodies are able to recognize a specific sequence and that the recognition is not length-dependent.

K. Monomer-by-Monomer Synthesis of YGGFL and YPGGFL

In order to further demonstrate the operability of the invention, a 50 μm checkerboard pattern of alternating YGGFL and YPGGFL was synthesized on a substrate using techniques like those set forth above. The resulting fluorescence plot showed that the antibody was clearly able to recognize the YGGFL sequence and did not bind significantly at the YPGGFL regions.

L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody

Using techniques similar to those set forth above, an array of 16 different amino acid sequences (replicated four times) was synthesized on each of two glass substrates. The sequences were synthesized by attaching the sequence NVOC-GFL across the entire surface of the slides. Using a series of masks, two layers of amino acids were then selectively applied to the substrate. Each region had dimensions of 0.25 cm x 0.0625 cm. The first slide contained amino acid sequences containing only L amino acids while the second slide contained selected D amino acids. Figs. 14A and 14B illustrate a map of the various regions on the first and second slides, respectively. The patterns shown in Figs. 14A and 14B were duplicated four times on

each slide. The slides were then exposed to the Herz antibody and fluorescein-labeled goat anti-mouse.

A fluorescence plot of the first slide, which contained only L amino acids showed red areas (indicating strong binding, i.e., 149,000 counts or more) and black areas (indicating little or no binding of the Herz antibody, i.e., 20,000 counts or less). The sequence YGGFL was clearly most strongly recognized. The sequences YAGFL and YSGFL also exhibited strong recognition of the antibody. By contrast, most of the remaining sequences showed little or no binding. The four duplicate portions of the slide were extremely consistent in the amount of binding shown therein.

A fluorescence plot of the D amino acid slide indicated that strongest binding was exhibited by the YGGFL sequence. Significant binding was also detected to YaGFL, YsGFL, and YpGFL. The remaining sequences showed less binding with the antibody. Low binding efficiency of the sequence yGGFL was observed.

Table 6 lists the various sequences tested in order of relative fluorescence, which provides information regarding relative binding affinity.

Table 6

Apparent Binding to Herz Ab

L-a.a. Set	D-a.a. Set
YGGFL	YGGFL
YAGFL	YaGFL
YSGFL	YsGFL
LGGFL	YpGFL
FGGFL	fGGFL
YPGFL	yGGFL
LAGFL	faGFL
FAGFL	wGGFL
WGGFL	yaGFL
	fpGFL
	waGFL

VIII. Illustrative Alternative Embodiment

According to an alternative embodiment of the invention, the methods provide for attaching to the surface a caged binding member which in its caged form has a relatively low affinity for other potentially binding species, such as receptors and specific binding substances. Such techniques are more fully described in copending application Serial No. 404,920, filed September 8, 1989, and incorporated herein by reference for all purposes.

According to this alternative embodiment, the invention provides methods for forming predefined regions on a surface of a solid support, wherein the predefined regions are capable of immobilizing receptors. The methods make use of caged binding members attached to the surface to enable selective activation of the predefined regions. The caged binding members are liberated to act as binding members ultimately capable of binding receptors upon selective activation of the predefined

regions. The activated binding members are then used to immobilize specific molecules such as receptors on the predefined region of the surface. The above procedure is repeated at the same or different sites on the surface so as to provide a surface prepared with a plurality of regions on the surface containing, for example, the same or different receptors. When receptors immobilized in this way have a differential affinity for one or more ligands, screenings and assays for the ligands can be conducted in the regions of the surface containing the receptors.

The alternative embodiment may make use of novel caged binding members attached to the substrate. Caged (unactivated) members have a relatively low affinity for receptors of substances that specifically bind to uncaged binding members when compared with the corresponding affinities of activated binding members. Thus, the binding members are protected from reaction until a suitable source of energy is applied to the regions of the surface desired to be activated. Upon application of a suitable energy source, the caging groups labilize, thereby presenting the activated binding member. A typical energy source will be light.

Once the binding members on the surface are activated they may be attached to a receptor. The receptor chosen may be a monoclonal antibody, a nucleic acid sequence, a drug receptor, etc. The receptor will usually, though not always, be prepared so as to permit attaching it, directly or indirectly, to a binding member. For example, a specific binding substance having a strong binding affinity for the binding member and a strong affinity for the receptor or a conjugate of the receptor may be used to act as a bridge between binding members and receptors if desired. The method uses a receptor prepared such that the receptor retains its activity toward a particular ligand.

Preferably, the caged binding member attached to the solid substrate will be a photoactivatable biotin complex, i.e., a biotin molecule that has been chemically modified with photoactivatable protecting groups so that it has a significantly reduced binding affinity for avidin or avidin analogs than does natural biotin. In a preferred embodiment, the protecting groups localized in a predefined region of the surface will be removed upon application of a suitable source of radiation to give binding members, that are biotin or a functionally analogous compound having substantially the same binding affinity for avidin or avidin analogs as does biotin.

In another preferred embodiment, avidin or an avidin analog is incubated with activated binding members on the surface until the avidin binds strongly to the binding members. The avidin so immobilized on predefined regions of the surface can then be incubated with a desired receptor or conjugate of a desired receptor. The receptor will preferably be biotinylated, e.g., a biotinylated antibody, when avidin is immobilized on the predefined regions of the surface. Alternatively, a preferred embodiment will present an avidin/biotinylated receptor complex, which has been previously prepared, to activated binding members on the surface.

IX. Conclusion

The present inventions provide greatly improved methods and apparatus for synthesis of polymers on substrates. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. By way of example, the invention has been described primarily with reference to the use of photoremovable protective groups, but it will be readily recognized by those of skill in the art that sources of radiation other than light could also be used. For example, in some

embodiments it may be desirable to use protective groups which are sensitive to electron beam irradiation, x-ray irradiation, in combination with electron beam lithograph, or x-ray lithography techniques. Alternatively, the group could be removed by exposure to an electric current. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

C L A I M S

1. A method of preparing sequences on a substrate comprising the steps of:

a) exposing a first region of said substrate to an activator to remove a protective group;

b) exposing at least said first region to a first monomer;

c) exposing a second region to an activator to remove a protective group; and

d) exposing at least said second region to a second monomer.

2. The method as recited in claim 1 wherein said steps of exposing to an activator use an activator selected from the group consisting of ion beams, electron beams, gamma rays, x-rays, ultra-violet radiation, light, infra-red radiation, microwaves, electric currents, radiowaves, and combinations thereof.

3. The method as recited in claim 1 wherein said protective groups are photosensitive protective groups.

4. The method as recited in claim 1 wherein said steps of exposing to an activator are steps of applying light to selected regions of said substrate.

5. The method as recited in claim 1 wherein said first and the second monomers are amino acids.

6. The method as recited in claim 1 further comprising a step of screening sequences on said substrate for affinity with a receptor, said step of screening further comprising the step of exposing said substrate to said receptor and testing for the presence of said receptor in said first and said second region.

7. The method as recited in claim 6 wherein said receptor is an antibody.

8. The method as recited in claim 1 wherein said substrate is selected from the group consisting of polymerized Langmuir Blodgett film, functionalized glass, germanium, silicon, polymers, (poly)tetrafluoroethylene, polystyrene, gallium arsenide, and combinations thereof.

9. The method as recited in claim 1 wherein said protective group is selected from the group consisting of ortho-nitrobenzyl derivatives, 6-nitroveratryloxy-carbonyl, 2-nitrobenzyloxycarbonyl, cinnamoyl derivatives, and mixtures thereof.

10. The method as recited in claim 1 wherein said first and second regions each have total areas of less than 1 cm^2.

11. The method as recited in claim 1 wherein said first and second regions each have total areas of between about 1 μm^2 and 10,000 μm^2.

12. The method as recited in claim 4 wherein said light is monochromatic coherent light.

13. The method as recited in claim 1 wherein said steps of exposing to an activator are carried out with a solution in contact with said substrate.

14. The method as recited in claim 13 wherein said solution further comprises said first or said second monomer.

15. The method as recited in claim 6 wherein said receptor further comprises a marker selected from the group consisting of radioactive markers and fluorescent

markers and wherein said step of testing for the presence of the receptor is a step of detecting said marker.

16. The method as recited in claim 1 wherein the steps of exposing to an activator further comprise steps of:

a) placing a mask adjacent to said substrate, said mask having substantially transparent regions and substantially opaque regions at a wavelength of light; and

b) illuminating said mask with a light source, said light source producing at least said wavelength of light.

17. The method as recited in claim 1 wherein said steps are repeated so as to synthesize 10^3 or more different sequences on said substrate.

18. The method as recited in claim 1 wherein said steps are repeated so as to synthesize 10^6 or more different sequences on said substrate.

19. A method of synthesizing a plurality of chemical sequences, said chemical sequences comprising at least a first and a second monomer, comprising the steps of:

a) at a first region on a substrate having at least a first and a second region, said first and said second region comprising a substrate protective group, activating said first region to remove said substrate protective group in said first region;

b) exposing said first monomer to said substrate, said first monomer further comprising a first monomer protective group, said first monomer binding at said first region;

c) activating said second region to remove said substrate protective group in said second region;

d) exposing said second monomer to said substrate, said second monomer further comprising a second monomer protective group, said second monomer binding at said second region;

e) activating said first region to remove said first monomer protective group;

f) exposing a third monomer to said substrate, said third monomer binding at said first region to produce a first sequence;

g) activating said second region to remove said second monomer protective group; and

h) exposing a fourth monomer to said substrate, said fourth monomer binding at said second region to produce a second sequence, said second sequence different from said first sequence.

20. A method of synthesizing a plurality of chemical sequences, said chemical sequences comprising at least a first and a second monomer, comprising the steps of:

a) on a substrate having at least a first and a second region deactivating said first region to provide a first protective group in said first region;

b) exposing said first monomer to said substrate, said first monomer binding at said second region;

c) removing said protective group in said first region;

d) deactivating said second region to provide a second protective group in said second region;

e) exposing said second monomer to said substrate, said second monomer binding at said first region;

f) removing said protective group in said second region;

g) deactivating said first region to provide a protective group in said first region;

h) exposing a third monomer to said substrate, said third monomer binding at said second region to produce a first sequence;

i) removing said protective group in said first region; and

j) exposing a fourth monomer to said substrate, said fourth monomer binding at said first region to produce a second sequence, said second sequence different than said first sequence.

21. A method of synthesizing at least a first polymer sequence and a second polymer sequence on a substrate, said first polymer sequence having a different monomer sequence from said second polymer sequence, comprising the steps of:

a) inserting a first mask between said substrate and an energy source, said mask having first regions and second regions, said first regions permitting passage of energy from said source, said second regions blocking energy from said source;

b) directing energy from said source at said substrate, said energy removing a protective group from first portions of said first polymer under said first regions of said first mask;

c) exposing a second portion of said first polymer to said substrate to create a first polymer sequence;

d) inserting a second mask between said substrate and said energy source, said second mask having first regions and second regions;

e) directing energy from said source at said substrate, said energy removing said protective group under said first regions of said second mask from first portions of said second polymer; and

f) exposing a second portion of said second polymer to said substrate, said second portion of said

second polymer binding with said first portion of said second polymer to create a second polymer sequence.

22. A method of screening a plurality of amino acid sequences for binding with a receptor comprising the steps of:

a) on a glass plate having at least a first surface, said at least a first surface comprising a photoprotective material selected from the group consisting of nitroveratryloxy carbonyl and nitrobenzyloxy carbonyl, reacting said at least a first surface with t-butoxycarbonyl for storage, said glass plate substantially transparent to at least ultraviolet light;

b) exposing said at least a first surface to TFA to remove said t-butoxycarbonyl;

c) placing said glass plate on a reactor, said reactor comprising a reactor space, said at least a first surface exposed to said reactor space;

d) placing a mask at a first position on said glass plate, said mask comprising first locations and second locations, said first locations substantially transparent to at least ultraviolet light and said second locations substantially opaque to at least ultraviolet light, said second locations comprising a light blocking material on a first surface of said mask, said first surface of said mask placed in contact with said glass plate;

e) filling said reactor space with a reaction solution;

f) illuminating said mask with at least ultraviolet light, said ultraviolet light removing said photoprotective material from said at least a first surface of said glass plate under said first locations of said mask;

g) exposing said first surface to a first amino acid, said first amino acid binding to regions of said at least a first surface from which said photoprotective material was removed, said first amino acid comprising said photoprotective group at a terminus thereof;

h) placing a mask in contact with said glass plate at a second position;

i) illuminating said mask with at least ultraviolet light, said ultraviolet light removing said photoprotective material from said at least a first surface of said glass plate under said first locations of said mask;

j) exposing said at least a first surface to a second amino acid, said second amino acid binding to regions of said at least a first surface from which said photoprotective material was removed, said second amino acid comprising said photoprotective group at a terminus thereof;

k) placing a mask in contact with said glass plate at a third position;

l) illuminating said mask with at least ultraviolet light, said ultraviolet light removing said photoprotective material from said at least a first surface of said glass plate under said first locations of said mask;

m) exposing said at least a first surface to a third amino acid, said third amino acid binding to regions of said at least a first surface from which said photoprotective material was removed;

n) placing a mask in contact with said glass plate at a fourth position;

o) illuminating said mask with at least ultraviolet light, said ultraviolet light removing said photoprotective material from said at least a first surface of said glass plate under said first locations of said mask;

p) exposing said at least a first surface to a fourth amino acid, said fourth amino acid binding to regions of said at least a first surface from which said photoprotective material was removed, said at least a first surface comprising at least first, second, third, and fourth amino acid sequences;

q) exposing said at least a first surface to an antibody of interest, said antibody of interest binding more strongly to at least one of said first, said second, said third, or said fourth amino acid sequences;

r) exposing said at least a first surface to a receptor, said receptor recognizing said antibody of interest and binding at multiple locations thereof, said receptor comprising fluorescein;

s) exposing said at least a first surface to light, said first surface fluorescing in at least a region where said more strongly bound amino acid sequence is located; and

t) detecting and recording fluoresced light intensity as a function of location across said at least a first surface.

23. A method of identifying at least one peptide sequence for binding with a receptor comprising the steps of:

a) on a substrate having a plurality of polypeptides, each having a photoremovable protective group, irradiating first selected polypeptides to remove said protective group;

b) contacting said polypeptides with a first amino acid to create a first sequence, second polypeptides on said substrate comprising a second sequence; and

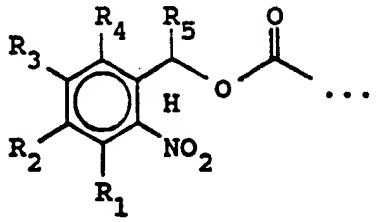
c) identifying which of said first or said second sequence binds with said receptor.

24. Apparatus for preparation of a plurality of polymers comprising:

a) a substrate with a surface, said surface comprising a reactive portion, said reactive portion activated upon exposure to an energy source so as to react with a monomer; and

b) means for selectively protecting and exposing portions of said surface from said energy source.

25. Apparatus as recited in claim 24 wherein said reactive portion further comprises a protective group, said protective group of the form:



where R_1 is alkoxy, alkyl, halo, aryl, alkenyl, or hydrogen; R_2 is alkoxy, alkyl, halo, aryl, nitro, or hydrogen; R_3 is alkoxy, alkyl, halo, nitro, aryl, or hydrogen; R_4 is alkoxy, alkyl, hydrogen, aryl, halo, or nitro; and R_5 is alkyl, alkynyl, cyano, alkoxy, hydrogen, halo, aryl, or alkenyl.

26. Apparatus as recited in claim 24 wherein said reactive portion further comprises linker molecules.

27. Apparatus as recited in claim 26 wherein said linker molecules are selected from the group consisting of ethylene glycol oligomers, diamines, diacids, amino acids, and combinations thereof.

28. Apparatus as recited in claim 24 wherein said means for selectively protecting further comprises a mask.

29. Apparatus as recited in claim 24 wherein said means for selectively protecting further comprises a light valve.

30. Apparatus as recited in claim 24 wherein said energy source is a light source.

31. Apparatus as recited in claim 24 wherein said reactive portion further comprises a composition selected from the group consisting of nitroveratryloxy carbonyl, nitrobenzyloxy carbonyl, dimethyl-dimethoxybenzyloxy carbonyl, 5-bromo-7-nitroindolinyl, hydroxy-2-methyl cinnamoyl, and 2-oxymethylene anthraquinone.

32. Apparatus for preparation of a substrate having a plurality of amino acid sequences thereon, said apparatus comprising:

a) a substrate with a surface;

b) a protective group on said surface, said protective group removable upon exposure to an energy source, said energy source selected from the group consisting of light, electron beams, and x-ray radiation;

c) means for directing said energy source at selected locations on said surface; and

d) means for exposing amino acids to said surface for binding to said surface.

33. Apparatus for screening polymers comprising a substrate with a surface, said surface comprising at least two predefined regions, said predefined regions containing different monomer sequences thereon, said predefined regions each occupying an area of less than about 0.1 cm^2.

34. Apparatus as recited in claim 33 wherein said area is less than about 0.01 cm^2.

35. Apparatus as recited in claim 33 wherein said area is less than 10000 μm^2.

36. Apparatus as recited in claim 33 wherein said area is less than about 100 μm^2.

37. Apparatus as recited in claims 33, 34, 35, or 36 wherein said monomer sequences are substantially pure within said predefined regions.

38. A substrate for screening for biological activity, said substrate comprising 10^3 or more different ligands on a surface thereof in predefined regions.

39. A substrate as recited in claim 38 wherein said substrate comprises 10^4 or more different ligands in predefined regions.

40. A substrate as recited in claim 38 wherein said substrate comprises 10^5 or more different ligands in predefined regions.

41. A substrate as recited in claim 38 wherein said substrate comprises 10^6 or more different ligands in predefined regions.

42. A substrate as recited in claims 38, 39, 40, or 41 wherein the ligands are peptides.

43. A substrate as recited in claim 33 wherein said ligands are substantially pure within said predefined regions.

44. Apparatus for screening for biological activity comprising:

a) a substrate comprising a plurality of polymer sequences, said polymer sequences attached to a surface of said substrate at known locations on said substrate, each of said sequences occupying an area of less than about 0.1 cm^2;

b) means for exposing said substrate to a receptor, said receptor marked with a fluorescent marker, said receptor binding with at least one of said sequences; and

c) means for detecting a location of said fluorescent marker on said substrate.

45. Apparatus for forming a plurality of polymer sequences comprising:

a) a substrate, said substrate having at least a first surface and a second surface, said second surface comprising a photoremovable protective material, said substrate substantially transparent to at least light of a first wavelength;

b) a reactor body, said reactor body having a mounting surface with a reaction fluid cavity therein, said second surface maintained in a sealed relationship with said mounting surface; and

c) a light source for producing light of at least said first wavelength and directed at a surface of said substrate.

46. Apparatus for detection of fluorescent marked regions on a substrate comprising:

a) a light source for directing light at a surface of said substrate;

b) a means for detecting light fluoresced from said surface in response to said light source;

c) means for translating said substrate from a first position to a second position; and

d) means for storing fluoresced light intensity as a function of location on said substrate, said means for storing connected to said means for translating and said means for detecting.

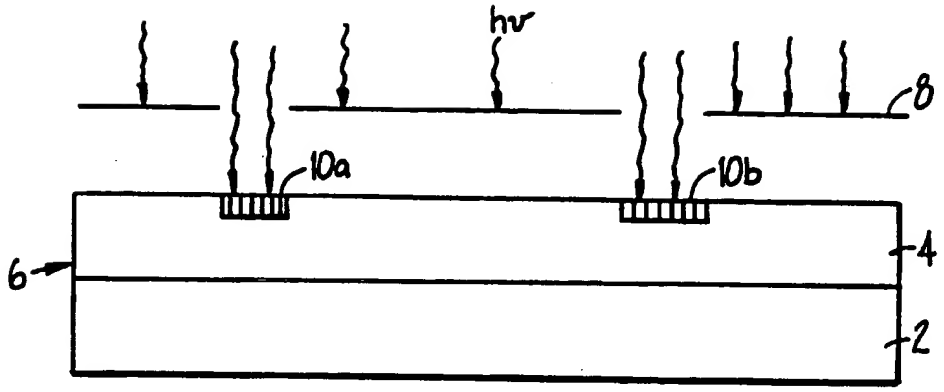


FIG._1.

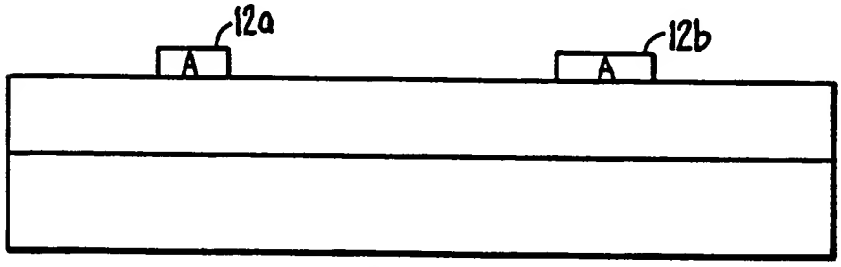


FIG._2.

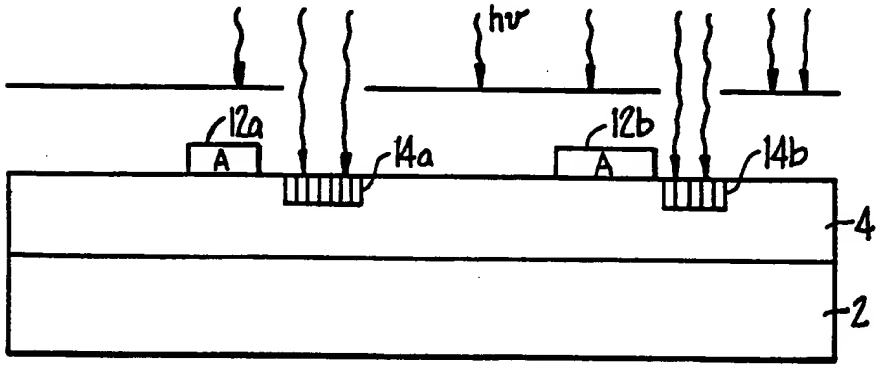


FIG._3.

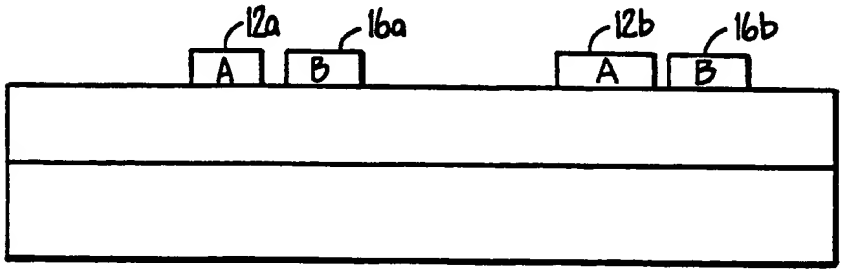


FIG._4.

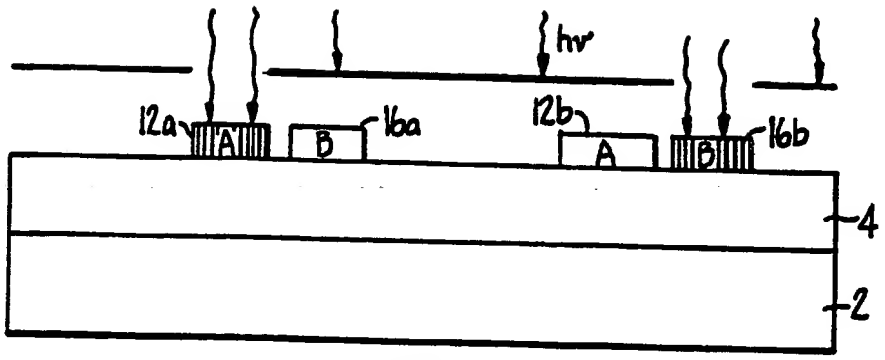


FIG._5.

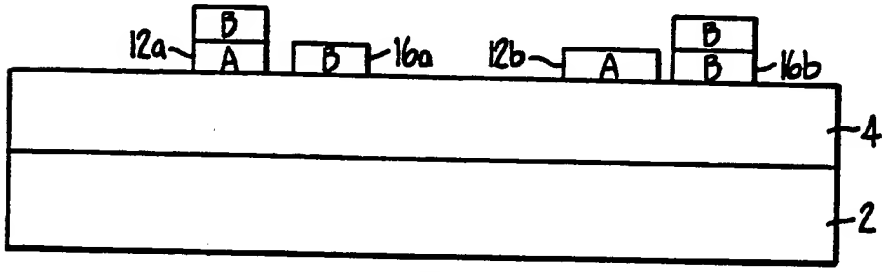


FIG._6.

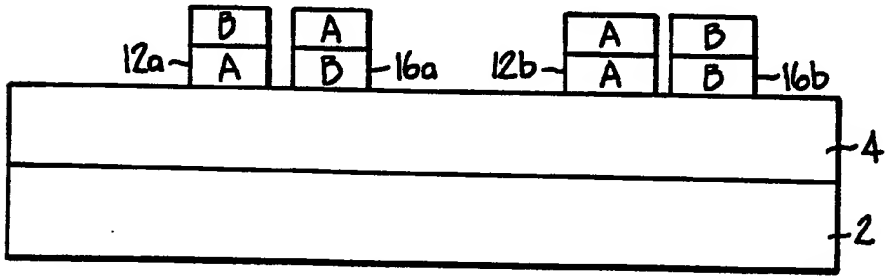


FIG._7.

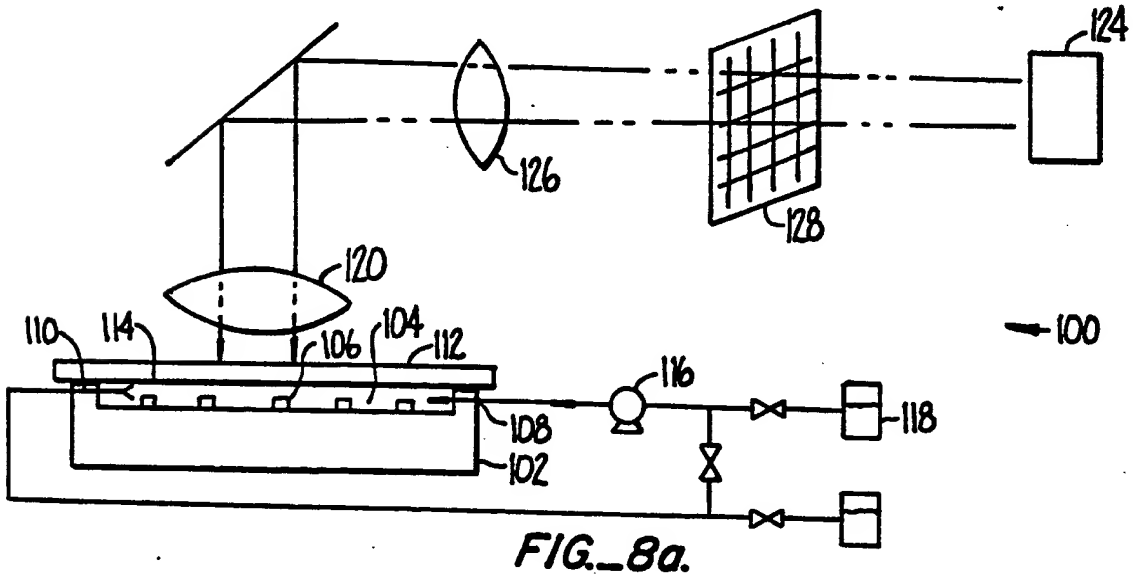


FIG._8a.

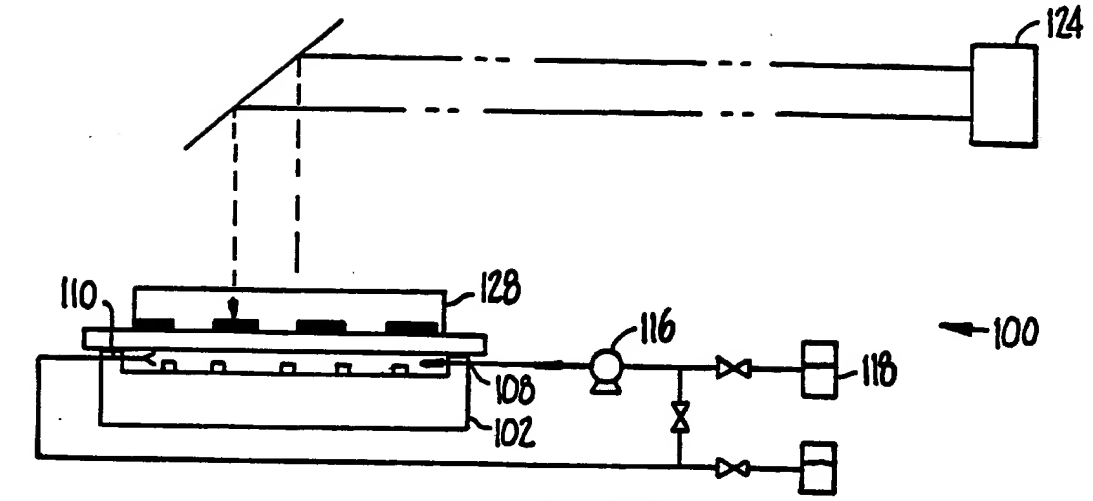
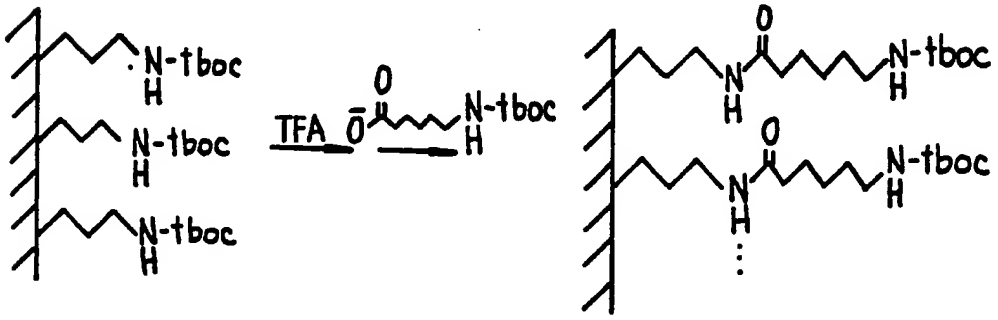
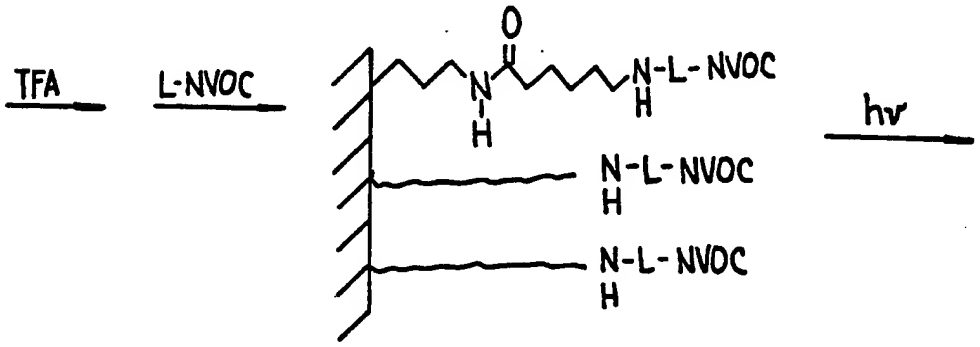


FIG._8b.





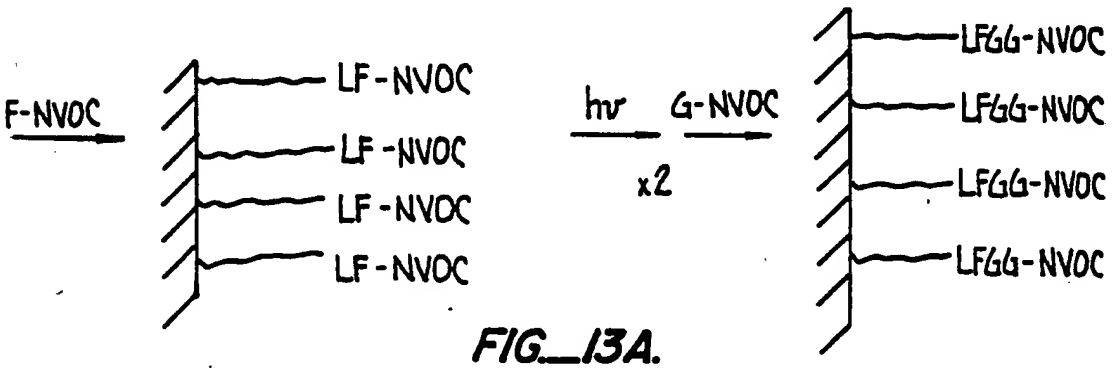


FIG._13A.

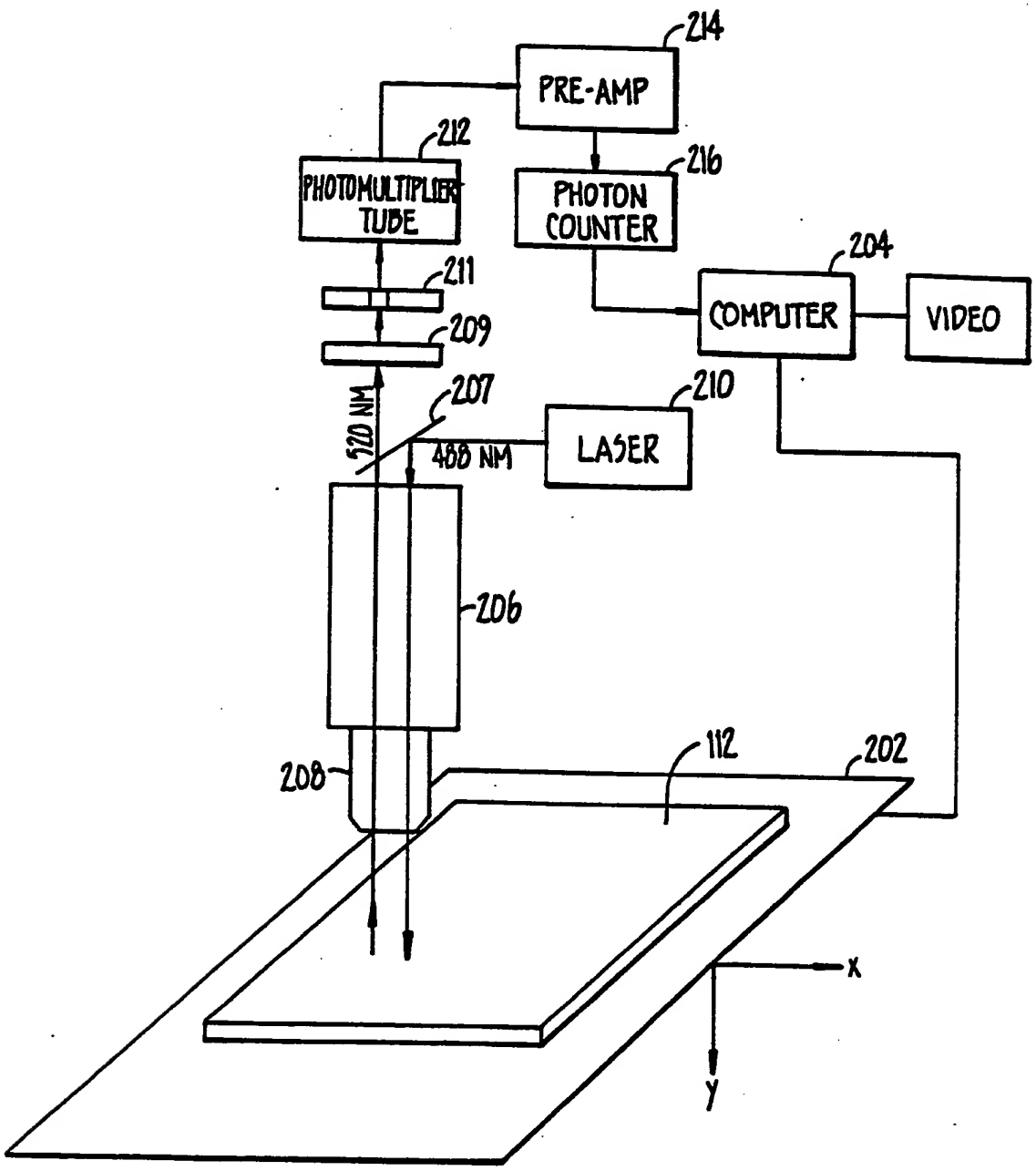

214
PRE-AMP
212
PHOTOMULTIPLIER TUBE
216
PHOTON COUNTER
204
COMPUTER
VIDEO
211
209
207
520 NM
488 NM
210
LASER
206
208
112
202
X
Y

FIG_9.

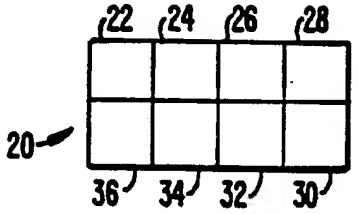


FIG._10A.

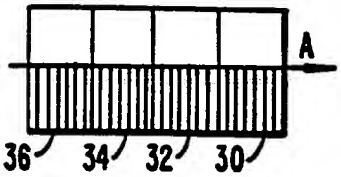


FIG._10B.

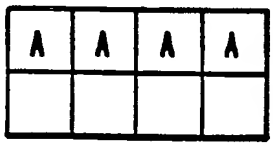


FIG._10C.

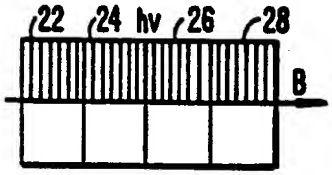


FIG._10D.

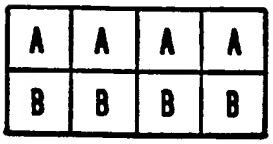


FIG._10E.

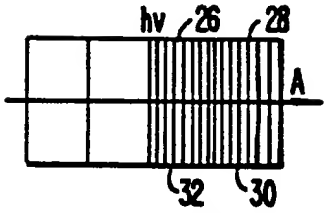


FIG._10F.

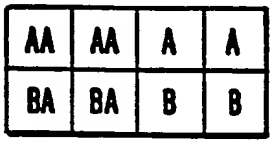


FIG._10G.

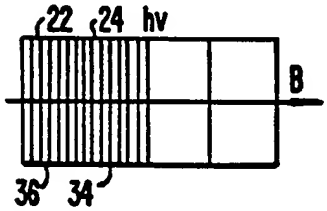


FIG._10H.

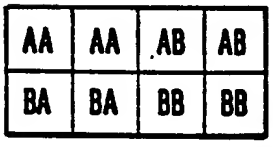


FIG._10I.

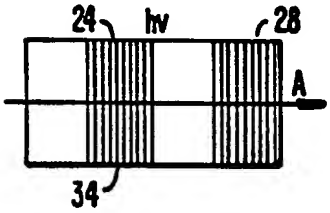


FIG._10J.

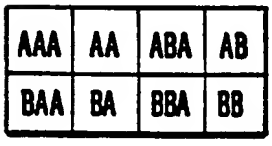


FIG._10K.

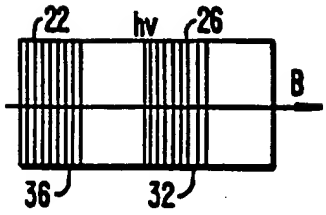


FIG._10L.

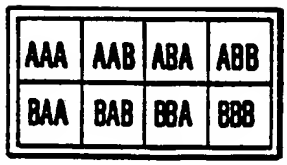


FIG._10M.

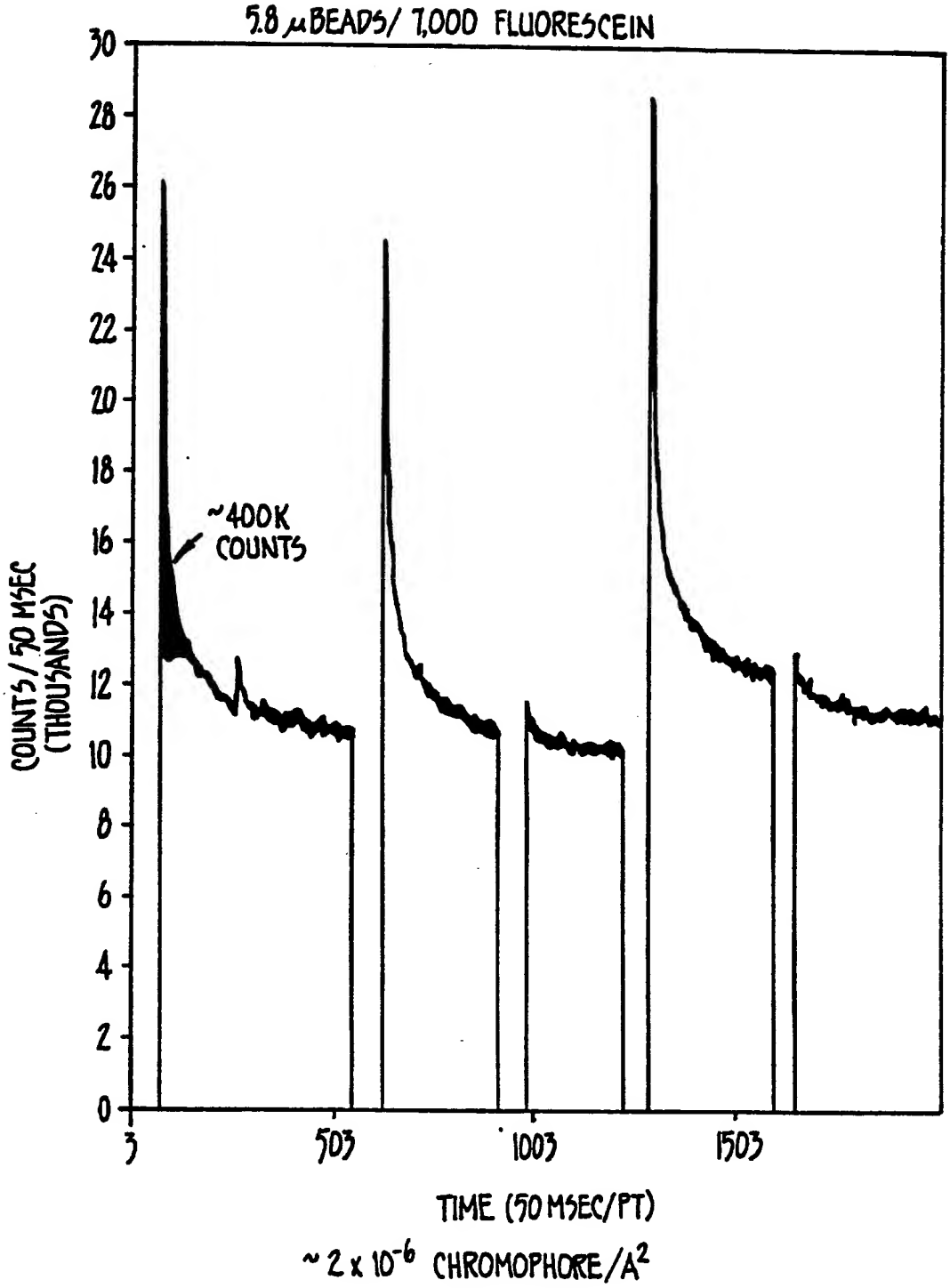

5.8 μBEADS/ 7,000 FLUORESCEIN
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
~400K COUNTS
COUNTS/50 MSEC (THOUSANDS)
3
503
1003
1503
TIME (50 MSEC/PT)
~ 2 x 10⁻⁶ CHROMOPHORE/A²

FIG._IIA.

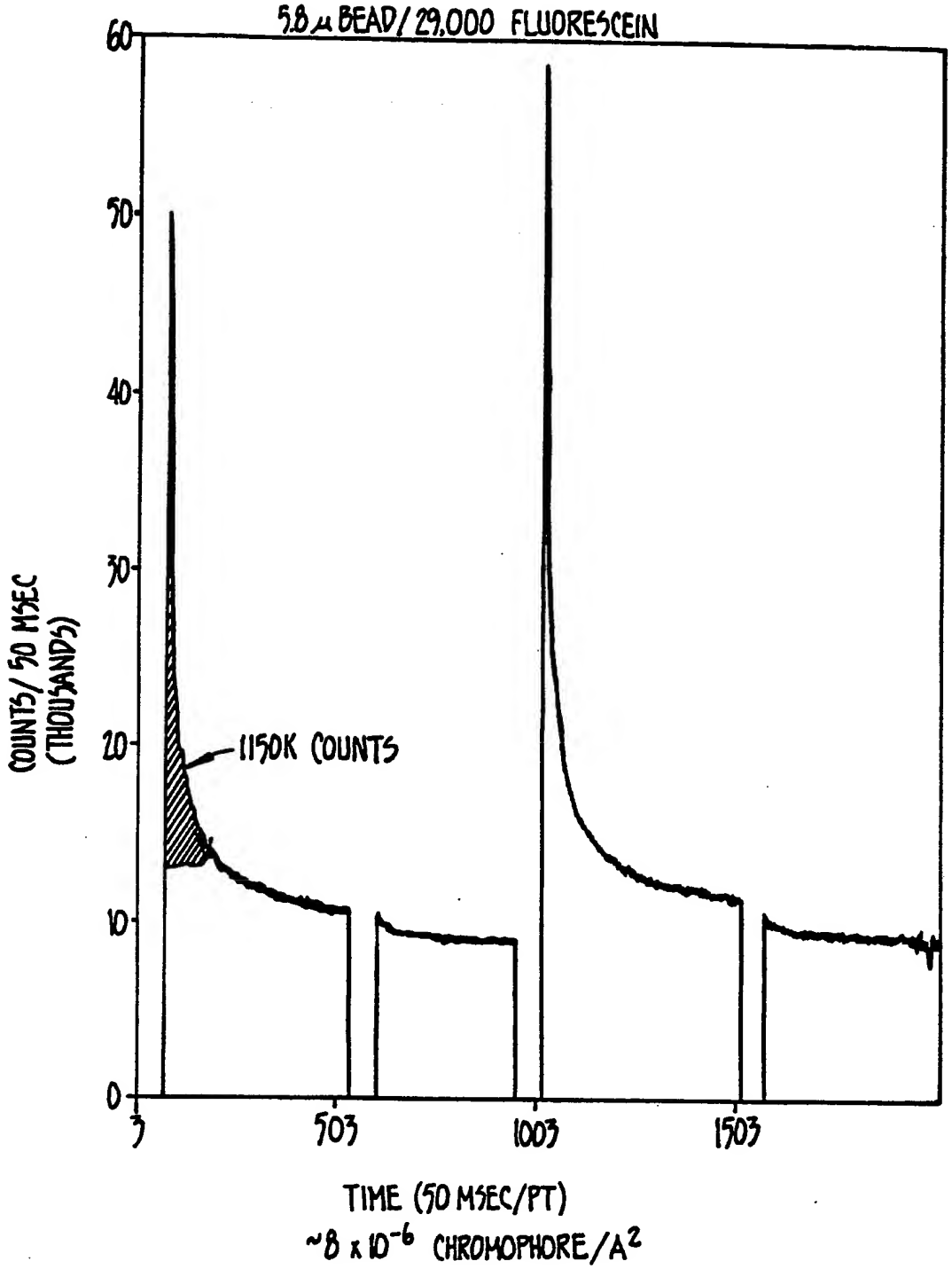

5.8 μ BEAD/ 29,000 FLUORESCEIN
60
50
40
30
20
10
0
COUNTS/ 50 MSEC (THOUSANDS)
1150K COUNTS
3
503
1003
1503
TIME (50 MSEC/PT)
~8 x 10⁻⁶ CHROMOPHORE/A²

FIG._11B.

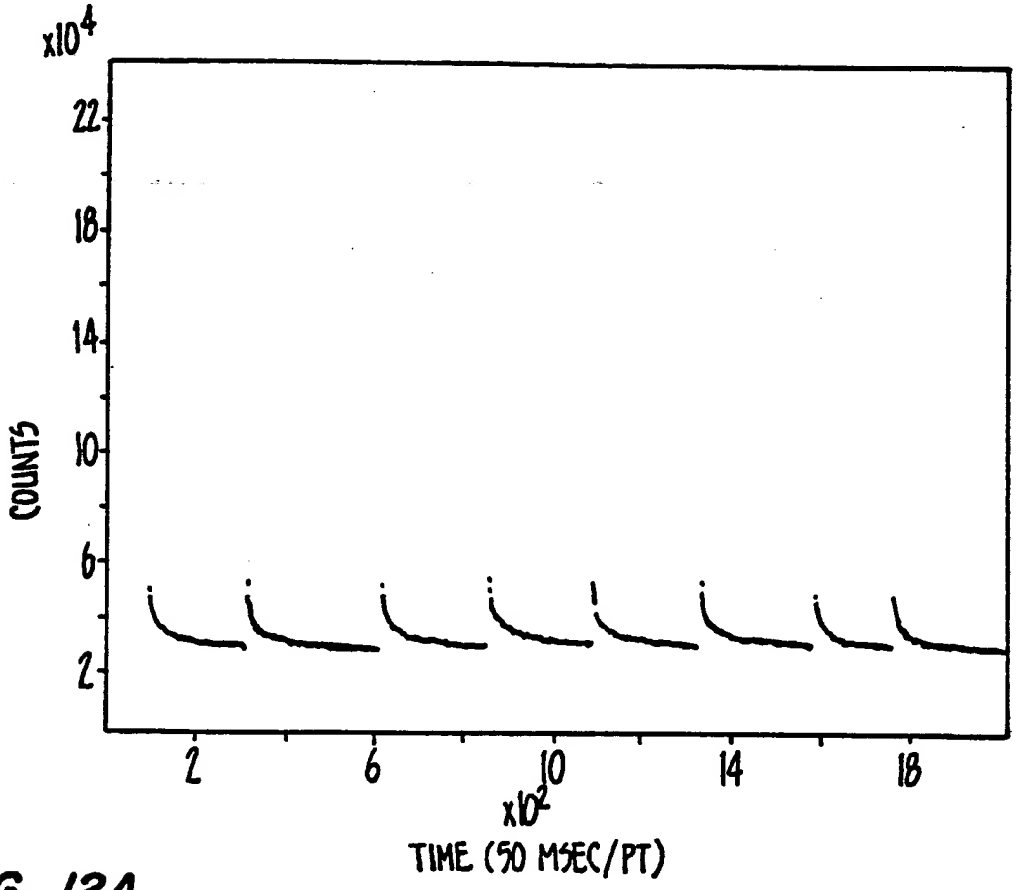


FIG._12A.

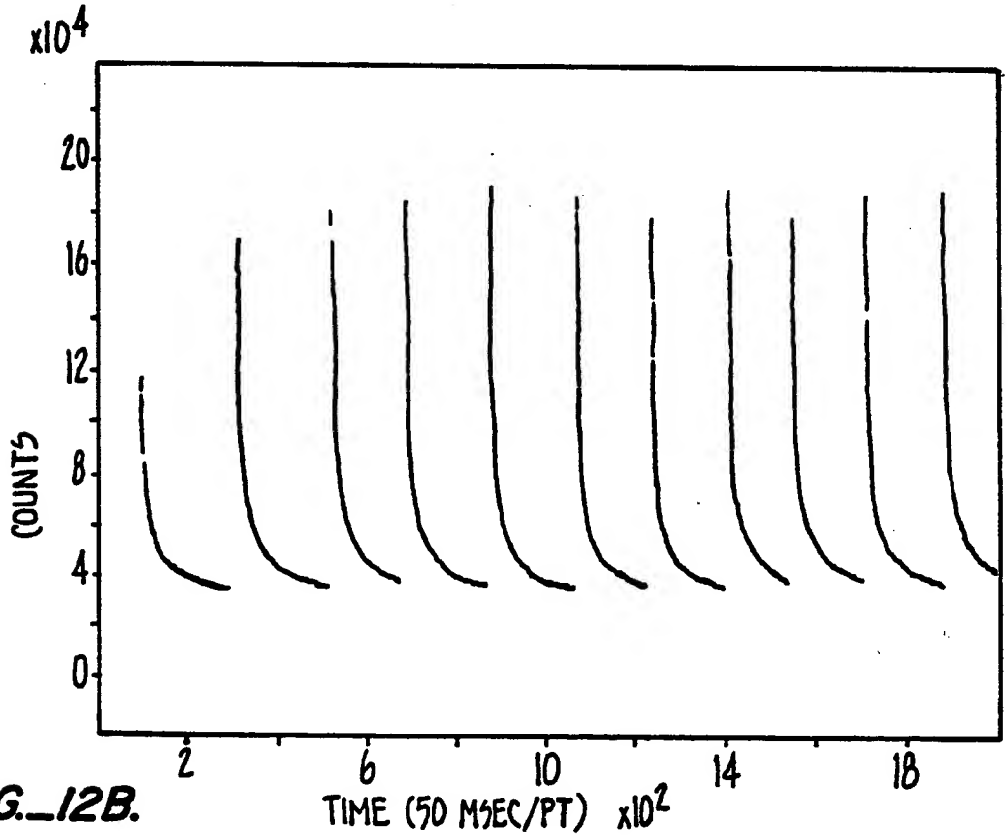


FIG._12B.

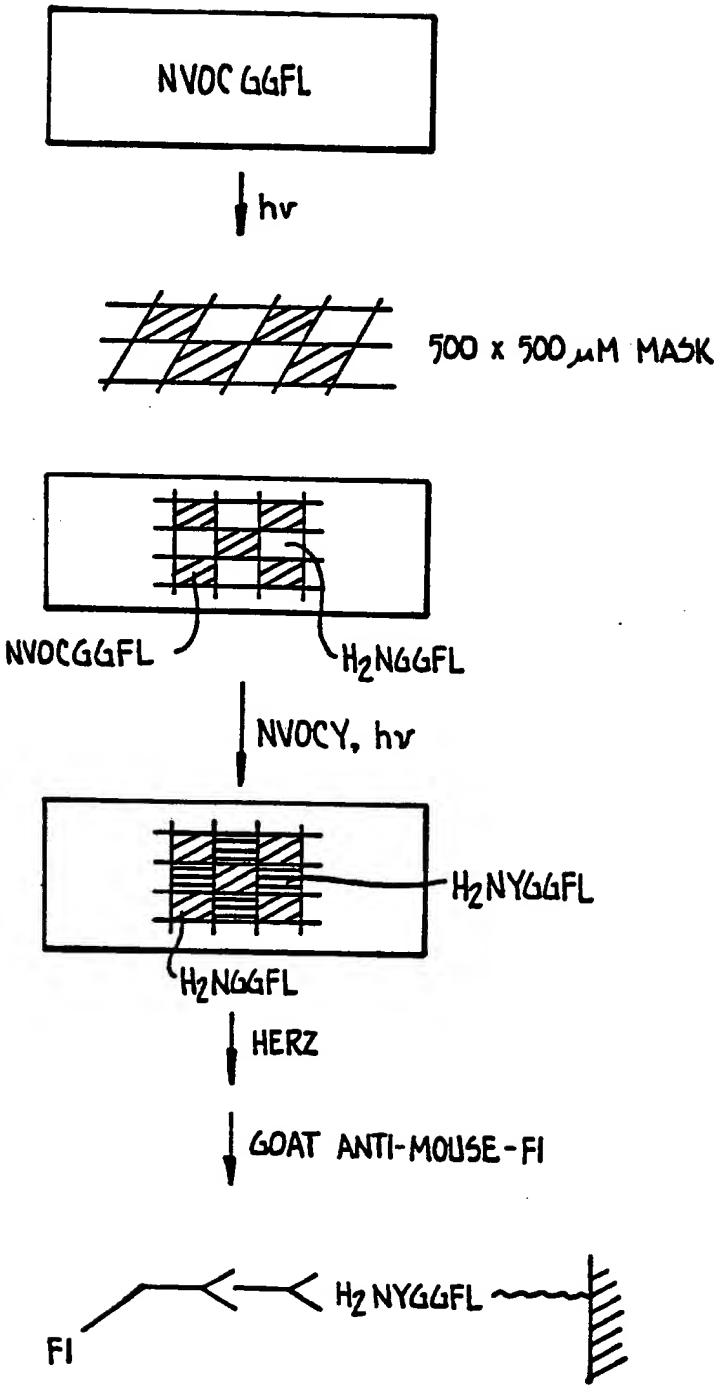

NVOC GGFL
hv
500 x 500 μM MASK
NVOCGGFL
H2NGGFL
NVOCY, hv
H2NYGGFL
H2NGGFL
HERZ
GOAT ANTI-MOUSE-FI
FI
H2 NYGGFL

FIG_13B

P	A	S	G	
LPGFL	LAGFL	LSGFL	LGGFL	L
FPGFL	FAGFL	FSGFL	FGGFL	F
WPGFL	WAGFL	WSGFL	WGGFL	W
YPGFL	YAGFL	YSGFL	YGGFL	Y

L SET

FIG_14A.

P	a	s	G	
YpGFL	YaGFL	YsGFL	YGGFL	Y
fpGFL	faGFL	fsGFL	fGGFL	f
wpGFL	waGFL	wsGFL	wGGFL	w
ypGFL	yaGFL	ysGFL	yGGFL	y

D SET

FIG_14B.

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 90/00081

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 1/04, 17/06, 17/14, B 01 J 19/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, B 01 J

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, volume 110, no. 9, 27 February 1989, (Columbus, Ohio, US), V.K. Haridasan et al.: "Peptide synthesis using photolytically cleavable 2-nitrobenzyloxycarbonyl protecting group", see page 707, abstract 76031w, & Proc. Indian Natl. Sci. Acad., Part A 1987, 53(6), 717-28 (Eng).	1-5,9
	--	
A	Chemical Abstracts, volume 100, no. 17, 23 April 1984, (Columbus, Ohio, US), W. Stueber et al.: "Synthesis and photolytic cleavage of bovine insulin B22-30 on a nitrobenzoylglycyl-poly (ethylene glycol) support", see page 700, abstract 139591v, & Int. J. pept. Protein Res. 1983, 22(3), 277-83 (Eng).	1
	--	
	./.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th September 1990	18 OCT. 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ISS T. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	IBM Technical Disclosure Bulletin, volume 9. no. 11, April 1967, M.F. Levy: "Preparing additive printed circuits", page 1473, see the whole article	1
A	Chemical Abstracts, volume 93, no. 22, 1 December 1980, (Columbus, Ohio, US), M. Gazard et al.: "Lithographic technique using radiation-induced grafting of acrylic acid into poly (methyl methacrylate) films", see page 585, abstract 213252r & Polym. Eng. Sci. 1980, 20(16), 1069-72 (Eng).	1
A	J. Vac. Sci. Technol., volume B1, no. 4, October-December 1983, American Vacuum Society, M. Morita et al.: "Direct pattern fabrication on silicone resin by vapor phase electron beam polymerization", page 1171 see the whole article	1
P,X	EP, A, 0328256 (OWENS-CORNING) 16 August 1989 see the whole text, especially example 12	38-43

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9000081 SA 37813

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/10/90 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0328256	16-08-89	JP-A- 2006750	10-01-90